GROWTH AND METABOLISM OF STRAIN L FIBROBLASTS WITH REFERENCE TO THE ACTIONS OF UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

by

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ABBREVIATIONS

ADP	Adenosine diphosphate
AIB	α-Aminoisobutyric acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BAL	British anti-Lewisite
Cyt.	Cytochrome
DNA	Deoxyribonucleic acid
2:4-DNP	2:4-Dinitrophenol
EMP	Embden-Meyerhof Pathway
FAD	Flavin adenine nucleotide
FP	Flavoprotein
FMN	Flavin mononucleotide
Fe(n.h.)	Non-heme iron
HMP	Hexose Monophosphate shunt Pathway
IOD	Iodoacetate
LA	Lactic acid
LDH	Lactic dehydrogenase
MDH	Malic dehydrogenase
NAD	Nicotinamide adenine nucleotide
NADP	Nicotinamide adenine nucleotide phosphate
PCA	Perchloric acid
Pi	Inorganic phosphate
P:O	Number of atoms of Pi incorporated into organic phosphates per atom of oxygen consumed
PVP	Polyvinyl Pyrrolidone
RNA	Ribonucleic acid
TCA	Trichloroacetic acid

I N T R O D U C T I O N

This study is an investigation into some aspects of mammalian cell growth and metabolism in vitro, with particular reference to the actions of uncouplers of oxidative phosphorylation. Oxidative phosphorylation is the mechanism by which the cell utilises the energy made available in biological oxidations, oxidative reactions being linked, or capable of being linked to the phosphorylation of adenosine diphosphate (ADP) by inorganic phosphate (Pi) according to the formal equation

$$AH_2 + B + ADP + Pi \rightleftharpoons A + BH_2 + ATP + H_2O$$

which is the sum of two reactions which are essentially irreversible

$$AH_2 + B \longrightarrow A + BH_2 \qquad (1)$$

$$ADP + Pi \longleftarrow ATP + H_2O \qquad (2)$$

The oxidative reaction (1) must deliver sufficient energy to drive reaction (2) to the right. Despite its fundamental importance, the mechanism of oxidative phosphorylation has not yet been described. Since the finding of Loomis and Lipmann (1948) that 2:4-Dinitrophenol (2:4-DNP) uncouples oxidation from respiratory chain phosphorylations, research into the actions of uncouplers has centred primarily on three types of biological systems - isolated mitochondria and sub units, tissue slices, and micro-organisms. To a lesser extent, ascites tumour cells and red blood cells have been used. With the exception of the work to be described, no investigation of any length appears to have been carried out on tissue culture cells. Of these approaches, the most important information on the mechanism of oxidative phosphorylation and its uncoupling has been derived from investigations of isolated mitochondria and sub units. As a very large number of reviews provide extensive coverage of both historical and recent developments (Lardy and Elvehjam, 1945; Lehninger, 1951, 1955; Chance and Williams, 1956; Slater, 1955, 1958, 1961, 1964; Lehninger and Wadkins, 1962), it is proposed to outline only the more important aspects here as an essential background to the work to be described.

P:0 RATIOS

Interest in respiratory chain phosphorylation may conveniently be regarded as dating from the finding of Belitzer and Tsibakowa (1939) and Ochoa (1940, 1941) that more than one atom of Pi was esterified for every atom of oxygen used by respiring cell free systems, and the inference that phosphorylation therefore occurred not only during dehydrogenation but during the further transport of H atoms or electrons along the respiratory chain to oxygen. Since then, attempts to elucidate the precise stoicheiometry of oxidative phosphorylations, and the sequence and identity of the respiratory carriers, have been pursued concurrently as of key importance in the study of oxidative phosphorylation. Despite the difficulties inherent in the first of these problems, particularly that of measuring the esterification of Pi in systems complicated by high kinase and ATPase activities, it is of note that the conclusion of Ochoa in 1943 that the complete oxidation of pyruvate by cell free extracts of cat heart muscle was accompanied by the formation of 15 adenosine triphosphates is still considered as being essentially correct. With the development of methods for isolating mitochondria with low ATPase activity from crude homogenate (Schneider and Hogeboom, 1950; Kennedy and Lehninger, 1948, 1949; Kielley and Kielley, 1951), the use of hexokinase-catalysed reactions to "trap" the ATP formed by transphosphorylation reactions (Loomis and Lipmann, 1948; Hunter, 1949), spectrophotometric methods for measuring esterified P by linking this with the phosphorylation of hexose monophosphate and NADH oxidation (Slater, 1953) and other techniques critically discussed by Slater (1964), generally accepted P:0 ratios for the five oxidative steps of the Citric Acid Cycle have now emerged. These are summarised overleaf (Hunter, 1951).

	REACTION	Hydrogen Carrier	P:0 Ratio	ATP Synthesised
(i)	Oxidative decarboxylation of pyruvate	NADH	3	3
(ii)	isocitric - oxalosuccinic	NADPH	3	3
(iii)	oxidative decarboxylation of a-ketoglutarate	NADH	3	3
	plus ATP synthesis from ADP + Pi	-	-	1
(iv)	succinic - fumaric	FPH	2	2
(v)	malic - oxalacetic	NADH	3	3
		TOTAL		15

A point of some importance is that in Reaction (iii) there is one substrate-linked phosphorylation, that is a phosphorylation linked to the reduction of NAD in contrast to the other phosphorylations which are linked to the oxidation of NADH or reduced flavoprotein and hence termed respiratory-chain phosphorylations (Hunter, 1949). The experimentally found values for P:0 ratios invariably differ from the whole numbers denoted above. This has been taken to indicate experimental error and the occurrence of side reactions, and there seems no reason to question the assumption that the whole number nearest the majority of experimentally determined ratios is the correct value. Slater (1964) has suggested that it is theoretically impossible to obtain precise results since physiological reactions hydrolysing ATP for the maintenance of mitochondrial structure and ionic composition cannot be eliminated.

THE ELECTRON TRANSPORT SYSTEM

Although it is generally recognised that the main components of the electron transport system are the dehydrogenases specific for each substrate, the pyridine nucleotides, the flavoproteins and the cytochromes, lack of detailed knowledge on the respiratory chain is one of the major obstacles to elucidation of the mechanism of oxidative phosphorylation. Largely as a result of the work done in Green's

laboratory (Green, 1959), it is now well established that the system is localised in the mitochondria in an organised spatial arrangement which allows a rapid flow of electrons from substrate to oxygen. Of the several components of the chain the position of the pyridine nucleotides (Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP)) is most clearly established, interest in these dating from the discovery of Harden and Young (1906) that low molecular weight compounds were involved in dehydrogenase reactions during glucose fermentation. The isolation and chemical definition of these was achieved largely through the work of Schlenk and von Euler (1936) and Warburg and Christian (1935, 1936) and it is now clear that a very large number of dehydrogenase reactions are linked to one or other of the pyridine nucleotides as the first carriers of hydrogen atoms (Mehler, 1948). In this respect, Green's latest paper (1964) in which he attempts to summarise the work done in his laboratory over the past decade, is of interest. In this he envisages the dehydrogenase enzyme complexes as sited on the outer mitochondrial wall, the respiratory chain sited predominantly on the inner wall and cristae, and the pyridine nucleotides as mobile carriers in the matrix between, acting as a link between the two systems. Although an attractive synthesis, this must be regarded as being highly speculative.

Evidence for flavoproteins in the respiratory chain is now irrefutable, and flavin-containing preparations from a wide variety of biological sources have been found to catalyse electron transfer from reduced pyridine nucleotides to the cytochromes (Dewan and Green, 1938; Straub, 1939; Horecker, 1950, Edelhoch, Haysishi and Teply, 1952). The identity of the flavoprotein which oxidises NADH is however still uncertain and Slater (1958) has critically discussed the marked differences between several of the preparations isolated with respect to inhibition by antimycin, acceptor specificity and even prosthetic group. Information from more recent experiments has not entirely resolved the question as to whether the

prosthetic group is Flavin adenine dinucleotide (FAD) (Ziegler, Green and Doeg, 1959; Ringler, Minakami and Singer, 1960; King and Howard, 1960) or Flavin mononucleotide (FMN) (Huennekens, Felton, Rao and Mackler, 1961; King and Howard, 1962) or some other group (Mahler, Sarkar, Vernon and Alberty, 1952). Ernster (1961) has concluded that mitochondria from most animal tissues seem to contain on a functional basis at least four different types of flavoenzymes, three being specific for NADH and one for NADPH. Of the NADH specific flavoenzymes, one is an a-lipoyl dehydrogenase which functions in the oxidation of a-keto acids and may therefore be regarded as an NAD reductase (Massey, 1958), one is an NADH-cytochrome c reductase as found by Edelhoch et al (1952) while the other is an NADH dehydrogenase which is part of the main respiratory chain. In this respect Singer, Minakami and Ringler (1961) claim to have solubilised and isolated the latter flavoenzyme from particulate electron transport particles fractionated from heart mitochondria.

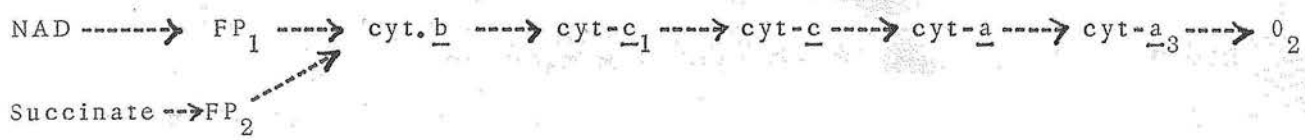
The labile nature of the FAD prosthetic group of this enzyme suggested to these authors that the profoundly different properties of many of the preparations of other investigators could be explained by modification during preparation. The problem of identifying the enzyme, however, is underlined by the work of King, Howard and Wilson (1961) who isolated an enzyme from heart muscle preparation by identical techniques, but with properties which differ in most respects from that of Singer et al. Recently Merola, Coleman and Hansen (1963) have concluded that FMN is the acid extractable flavin of the respiratory chain NADH dehydrogenase but are not prepared to argue that this is the group on the intact enzyme. This view is supported by Cremona and Kearney (1964) who claim that the flavin moiety is FMN, and that the FAD found by others is due to impurities which can be separated at pH 10 but not at neutral pH. It is clear, therefore, that although flavoproteins are of obvious importance in transferring electrons

from the pyridine nucleotides to the cytochromes, agreement has not been reached on either the specificity or the relationships of these.

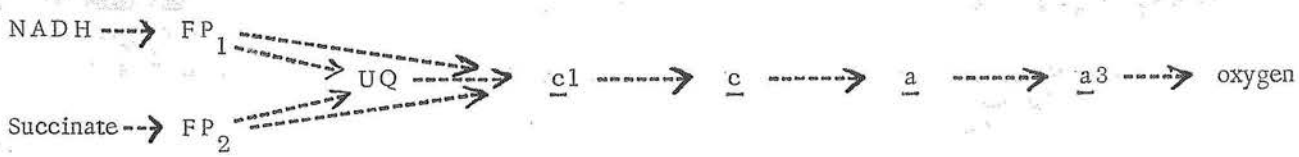
It has been recognised that the cytochromes play a fundamental role in respiration since 1925 when Keilin demonstrated absorption bands corresponding to three different heme proteins which he named cytochromes a, b and c. It was later shown that the a band was composed of two spectrophotometrically distinct entities which always occurred in constant proportions and could not be separated by physical means (Keilin and Hartree, 1939). These were designated a and a_3. Since cytochrome a_3 was autoxidisable and combined with carbon monoxide and cyanide causing a spectral shift, the authors concluded that this was in fact the enzyme which finally reacts with oxygen. Cytochromes a and a_3 are therefore generally regarded as the cytochrome oxidase complex. The cytochromes of the a group have however been thoroughly discussed by Lemberg (1961) with the conclusion that the relationship between a and a_3 is far from resolved. In fact the work of several authors (Ambe and Venkataraman, 1959; Yonetani, 1961) on purified preparations of cytochrome oxidase suggests that there is only a single cytochrome a molecule. Against this, more recent findings by Nicholls (1963) indicate that cytochromes a and a_3 are distinct and different entities, and Morrison and Horie (1964) claim to have prepared cytochrome a apparently free from cytochrome a_3. Cytochrome oxidase has been discussed to underline the fact that several aspects of the identity and interactions of the cytochromes are uncertain. Consideration of cytochromes of the c group show similar unresolved difficulties (Green, 1959; Lehninger and Wadkins, 1962). Early work on the sequence and identity of the cytochromes in the respiratory chain is thoroughly discussed by Slater (1958b).

Although Chance and Williams (1955) noted that in rat liver mitochondria, electron transport from NADH and succinate to cytochrome c appeared to be mediated by heme proteins similar spectrophotometrically to cytochrome b, it

was generally accepted on kinetic grounds that cytochrome b was not on the main pathway in mitochondria (Slater, 1958c). Re-assessment of the kinetics involved and the results of experiments with BAL (British anti-Lewisite) which inhibits the reduction of cytochrome c by NADH, but does not affect the transfer of electrons to cytochrome b or from cytochrome c to oxygen led Slater (1958) to question this conclusion. At present, the sequence and identity of respiratory carriers in the mitochondria of higher animals for which appropriate kinetic evidence exists, appears to be substantially that suggested by Lehninger and Wadkins (1962) and denoted below.

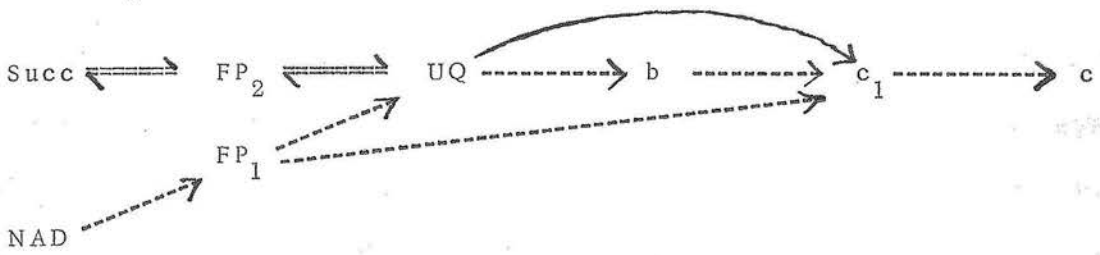


Certainly this is not the complete respiratory chain. Green (1964) for example suggests that it includes at least three species of non-heme iron protein. Furthermore, the position of ubiquinone is unresolved. Evidence that this functions as an oxido-reduction carrier is based on the fact that incubation of mitochondria with TCA Cycle intermediates under anaerobic conditions reduces it, while on aeration re-oxidation occurs (Crane, Hatefi, Lester and Widmer, 1957). Redfearn (1960, 1961) reviewing the kinetic aspects of the evidence, concluded that ubiquinone was not an obligatory carrier but may act as a branch pathway linking the flavoproteins with the cytochromes, as shown below.

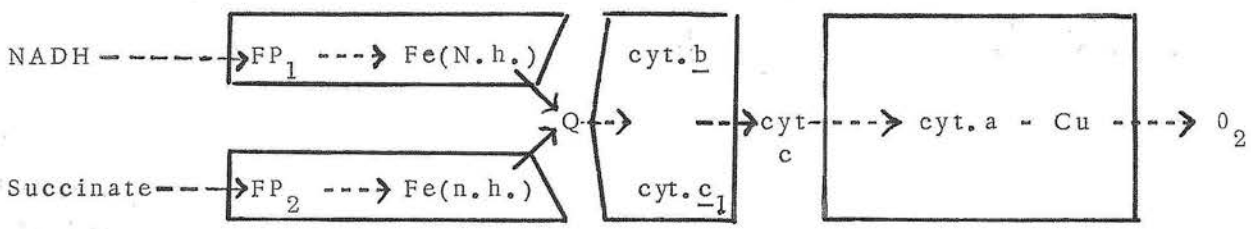


Chance (1961) has emphasised that there is no direct evidence that ubiquinone functions in the main respiratory chain, and postulates that it is primarily involved in the succinate linked reduction of NAD. On the basis of inhibitor studies, Slater, Colpa-Boonstra and Links (1961) conclude the ubiquinone occupies an important

position and promotes the entry of flavoproteins into the respiratory chain, NADH flavoproteins only being able to bypass ubiquinone if oxidised directly by cytochrome $\underline{c}_1$ according to the scheme shown below.



An obligatory main line role for ubiquinone between NADH flavoproteins and the cytochromes has been principally advocated by Green (1961) on the basis of the isolation and reconstitution of enzymic complexes of the respiratory chain. A development of this by Hatefi, Haavik, Fowler and Griffiths (1962) is shown below.



In this scheme NADH and the flavoproteins interact through non-heme iron carriers, with ubiquinone acting as a link between the flavoprotein and the cytochrome enzymic complexes. Green (1964) has recently reiterated this idea. A problem in the interpretation of experiments involving the reconstitution of segments of the respiratory chain is the relatively low specificity of interaction of isolated respiratory carriers and it is concluded that at present the position of ubiquinone is still in dispute.

LOCATION OF PHOSPHORYLATION STEPS IN THE RESPIRATORY CHAIN

Despite uncertainties on the precise sequence and identity of the respiratory carriers, considerable information has accrued on the location of the phosphorylation steps. These seem most likely to be sited between (i) NADH and cytochrome b (or ubiquinone), (ii) cytochrome b (or ubiquinone) and cytochrome c, and (iii) cytochrome c and oxygen. Calculations from P:0 ratios have been important in determining these spans. Consideration of the information on page 3 for example shows that in all cases where dehydrogenase reactions are pyridine nucleotide linked, the oxidation of the reduced co-enzyme is accompanied by three phosphorylations. Succinate oxidation which is not pyridine nucleotide linked, however, is accompanied by only two phosphorylations. On the assumption that oxidative phosphorylation is a quantised process, it can be argued that one phosphorylation is located between NADH and the point at which succinate enters the chain. As denoted in the various respiratory chain schemes on pages 7 and 8 , this is considered to be in the region of cytochrome b or ubiquinone. On the assumption that each step of the respiratory chain can only proceed when the free energy is negative, comparisons of the free energy available in each of the postulated spans, and that necessary for ATP synthesis are in general agreement with the scheme indicated above (Chance and Williams, 1956; Slater 1954, 1964). Observations on isolated regions of the chain also support the scheme. For example, P:0 ratios found on the oxidation of NADH as substrate (Lehninger, 1953) were approximately 3, while that found for the oxidation of cytochrome c (Nielson and Lehninger, 1954) was approximately 1. This is in agreement with two phosphorylations between NADH and cytochrome c, and one between cytochrome c and oxygen. Further information on the location of phosphorylation steps is provided by "crossover" experiments, first introduced by Chance and Williams (1955). This entails spectral examination of the respective states of reduction or oxidation of respiratory carriers on inhibition of respiration by antimycin or lack of ADP.

For example, when antimycin was added to mitochondria, cytochrome b and components of the chain before this were reduced while cytochromes c and a were oxidised. On relieving the inhibition by adding albumen, cytochrome b and prior components of the chain became more oxidised, and cytochromes c and a more reduced. There is thus a "crossover point" between cytochromes b and c on which the inhibitor acts. Chance and Williams found "crossover points" between cytochromes c and a, b and c, and NAD and flavoprotein.

Despite the weight of evidence pointing to phosphorylations occurring in the spans indicated above, some uncertainty remains and is underlined by the work of Ramirez (1954) and Ramirez and Mujica (1961, 1964) who present evidence on the basis of crossover experiments with intact cardiac muscle and heart sarcosomes for two phosphorylation sites between cytochrome c and oxygen (possibly in the cytochrome c to a and cytochrome a to oxygen couples). Howland (1963) has come to a similar conclusion from experiments on the oxidation of tetramethyl-p-phenylenediamine which is thought to enter the chain at cytochrome c. Slater (1958) has in fact calculated that there are no thermodynamic objections to two phosphorylation steps occurring between cytochrome c and oxygen.

THEORIES OF OXIDATIVE PHOSPHORYLATION

A: CHEMICAL THEORIES

The above considerations show that although there are probably three respiratory chain phosphorylations, in no case is the identity of the immediate hydrogen donor or acceptor known with certainty. Theories shall therefore be outlined in terms of general symbols rather than specific molecular entities.

Studies of substrate linked phosphorylation in which the substrate is phosphoglyceraldehyde, pyruvate or α-ketoglutarate, have shown that in each case a high energy intermediate is formed before the intervention of Pi and ADP, and that Pi reacts before ADP (Lipmann, 1939; Krimsby and Racker, 1955; Slater, 1953).

In 1953 Slater proposed that respiratory chain phosphorylation might be described in similar terms according to the formal equations -

$$AH_2 + B + C \rightleftharpoons A\sim C + BH_2 \quad \ldots\ldots\ldots\ldots\ldots (1)$$

$$A\sim C + ADP + Pi \rightleftharpoons A + C + ATP \quad \ldots\ldots\ldots\ldots\ldots (2)$$

$$\underline{\text{Sum}}\quad AH_2 + B + ADP + Pi \rightleftharpoons A + BH_2 + ATP \quad \ldots\ldots\ldots\ldots\ldots (3)$$

in which some substance C combines with the substrate A during the coupled oxido-reduction to form a bond between them having a high free energy of hydrolysis (1) which undergoes phosphorylation in the presence of Pi and ADP in a secondary reaction to form ATP (2). Reactions (1) and (2) may be envisaged as the sum reactions of several. The precedents from substrate linked phosphorylation suggest two rather different basic possibilities for reaction (1). These are

(a) Phosphoglyceraldehyde

$$B + C \rightleftharpoons BC$$

$$BC + AH_2 \rightleftharpoons A\sim C + BH_2$$

$$AH_2 + B+C \rightleftharpoons A\sim C + BH_2$$

(b) Pyruvate, α-ketoglutarate

$$AH_2 + B \rightleftharpoons A\sim BH_2$$

$$A\sim BH_2 + C \rightleftharpoons A\sim C + BH_2$$

$$AH_2 + B + C \rightleftharpoons A\sim C + BH_2$$

Two further alternatives are presented for reaction (2)

(a) Phosphoglyceraldehyde, pyruvate

$$A\sim C + Pi \rightleftharpoons A\sim P + C$$

$$A\sim P + ADP \rightleftharpoons A + ATP$$

$$A\sim C + ADP+Pi \rightleftharpoons A + C + ATP$$

(b) α-ketoglutarate

$$A\sim C + Pi \rightleftharpoons C\sim P + A$$

$$C\sim P + ADP \rightleftharpoons C + ATP$$

$$A\sim C + ADP + Pi \rightleftharpoons A+C+ATP$$

Slater (1961) concluded from the evidence available then, that each phosphorylative step in the respiratory chain could be described by the sequence

$$AH_2 + B + C \rightleftharpoons A\sim C + BH_2 \qquad (1)$$

$$A\sim C + Pi \rightleftharpoons C\sim P + A$$

$$C\sim P + ADP \rightleftharpoons C + ATP$$

$$AH_2 + B + ADP + Pi \rightleftharpoons A + BH_2 + ATP \qquad (3)$$

in which AH_2, B and C are different for each step. This might be regarded as the simplest formulation to explain the available information. It is far from conclusive and as Slater has stressed, the mechanism of oxidative phosphorylation may well be much more complex. The general theory that a high-energy intermediate is formed before the participation of Pi and ADP in that order is, however, widely supported at present (Lehninger, 1955, 1958; Boyer 1958; Lehninger and Wadkins, 1962; Hemker, 1963) and is compatible with the following experimental observations.

(1) Non-phosphorylating oxidations:

Although phosphorylations are tightly coupled to oxidations in intact mitochondria, preparations, such as the Keilin-Hartree heart muscle preparation in which mitochondrial structure is disrupted actively catalyse the aerobic oxidation of succinate and NADH in the absence of Pi or its esterification (Bonner, 1951, 1954). This suggests that Pi does not combine directly with a carrier before or during oxido-reduction as supposed by Low (1958), Grabbe (1958), Clarke and Todd (1960) and until recently, Ernster (1961).

(2) Effects of 2:4-DNP and Oligomycin:

In the course of their investigations into the uncoupling action of 2:4DNP, Loomis and Lipmann (1948) noted that the uncoupler could replace the Pi necessary for optimal respiration rates with kidney cyclophorase preparations. Although this conclusion has been challenged by Tepley (1949), Judah (1951) and Dawkins, Judah and Rees (1959) on the grounds that this does not occur when endogenous Pi is removed from the mitochondria, Borst and Slater (1961) have shown convincingly

that a substrate level phosphorylation was present in the experiments of these authors and that 2:4-DNP does in fact abolish the requirement for Pi in respiratory chain oxidation in intact mitochondria. This is entirely in agreement with the contention of Slater and Lewis (1954) that, since 2:4-DNP stimulated the oxidation of blowfly muscle mitochondria to a greater extent than addition of ADP and Pi did, the uncoupler acted by catalysing the hydrolysis of an intermediate compound at a point closer to the respiratory chain than the point of entry of Pi and ADP. Further circumstantial evidence comes from the work of Lardy and McMurray (1959) on oligomycin, a compound which inhibits phosphorylation, the P_{32}-ATP exchange and the exchange of O_2 between Pi and water and most significantly oxidation, this being considered to be a direct result of its compulsory coupling to phosphorylations. 2:4-DNP relieves the respiratory inhibition imposed by oligomycin by a mechanism which therefore does not involve the participation of either Pi or ADP. This is compatible with the idea that 2:4-DNP causes the splitting of an intermediate such as A~C in equation (1) so that intervention of Pi and ADP are unnecessary for respiration to proceed. Furthermore, such an action provides an explanation for the observation that 2:4-DNP induces ATPase activity (Hunter, 1951; Lardy and Wellman, 1953) for since the reaction of the postulated high energy intermediate A~C with ADP and Pi is reversible, hydrolysis of A C will lead to hydrolysis of ATP.

$$A\sim C + ADP + Pi \longleftarrow ATP + A + C$$

When 2:4-DNP is present, oligomycin does not inhibit respiration, but does inhibit the 2:4-DNP induced ATPase as it prevents the reaction between A, C and ATP (Lardy and McMurray, 1959).

(3) Exchange Reactions:

Over the past decade several reversible exchange reactions apparently related to oxidative phosphorylation have been reported for mitochondria and sub-mitochondrial particles. These include a rapid exchange of ^{18}O between water

and oxygen and the oxygen of Pi and ATP (Cohn and Drysdale, 1956, 1958; Chan, Lehninger and Enns, 1960), and ATP-^{32} Pi exchange which requires ADP as a component (Cooper and Lehninger, 1957) and an ATP-ADP exchange which occurs without the addition of Pi (Wadkins and Lehninger, 1958, 1960, 1963; Wadkins, 1961). On various grounds, including their sensitivity to 2:4-DNP and oligomycin, and the fact that the oxido-reduction state of the carriers profoundly influences the ATP-^{32}P exchange (Wadkins and Lehninger, 1959) and the ATP-ADP exchange (Wadkins, 1961), these reactions are considered to reflect the reversibility and multi-step nature of the coupling mechanism. Wadkins and Lehninger (1958) considered that the properties and component requirements of the reversible exchange reactions indicated a reaction sequence in which a high energy intermediate is formed first, followed by interaction of Pi and ADP in two separate steps. In terms of the symbols used here this may be represented

$$AH_2 + C + B \rightleftharpoons A\sim C + BH_2$$

$$A\sim C + Pi \rightleftharpoons C\sim P + A$$

$$C\sim P + ADP \rightleftharpoons ATP + C$$

The last step was considered as being reflected in the ATP-ADP exchange which showed no requirement for Pi, while the ATP-Pi exchange was considered to represent the sum of the last two steps, ADP being an essential component of this system. Boyer (1958) too, has concluded that the pattern of ^{18}O exchange is appropriate for a sequence in which Pi reacts before ADP.

More recently the above formulation has been modified (Wadkins and Lehninger, 1963) to take into account the isolation and behaviour of the enzyme (E) catalysing the ATP-ADP exchange, and an associated purified protein designated the M factor (Lehninger, 1961). In contrast to its activity in intact mitochondria, the ATP-ADP exchange enzyme is uninhibited by 2:4-DNP and oligomycin in its isolated state. These characteristics are restored on addition

to digitonin particles, this being promoted by the M factor, a specific protein isolated from mitochondrial extracts. Since the M factor itself does not confer 2:4-DNP sensitivity on the isolated enzyme but requires the presence of the particles, Lehninger has postulated that this factor is a component in a sequence and is necessary to combine specifically with an earlier catalyst in the sequence and that the exchange reaction is in equilibrium with a preceding DNP sensitive reaction. His postulated mechanism may thus be written -

$$AH_2 + M.E. \rightleftharpoons AH_2.M.E.$$

$$AH_2M.E. + B \rightleftharpoons A\sim M.E. + BH_2$$

$$A\sim M.E. + Pi \rightleftharpoons A + M.E.\sim P$$

$$M.E.\sim P + ADP \rightleftharpoons M.E. + ATP$$

This mechanism adequately explains the results of Wadkins and Lehninger's isolation and recombination experiments. The two coupling factors E and M are regarded as being bound together in intact mitochondria, but dissociating on preparation of the digitonin particles. In the intact mitochondria the complex M.E combines with reduced carrier, and is energised by oxidation to A~M.E which is in turn attacked by Pi to form P~M.E. P is then transferred to E within the complex before its reaction with ADP. The authors regard this only as a working hypothesis. The kinetics of isotopic exchanges are difficult to interpret as they may depend upon dissociation constants of intermediate enzyme substrate complexes, cofactor and substrate concentrations and necessary sequence of interacting components, factors which are uncertain in most experimental systems. Furthermore, it cannot be concluded that the ATP-ADP exchange participates at all three coupling sites of the respiratory chain. An additional difficulty in drawing firm conclusions from such experiments is that the properties of sub-mitochondrial particles may differ significantly depending upon their mode of preparation (Lehninger and Wadkins, 1962). It should be noted that Kulka and Cooper (1962) have presented evidence which conflicts with the ideas of

Wadkins and Lehninger. The kinetics of the ATP-ADP and ATP-Pi exchange reactions found by these authors cannot, in fact, be accounted for on the basis of Pi entering the mechanism two steps before ADP.

(4) Reversal of Respiratory Chain:

At present no convincing reports have appeared on the isolation of a high-energy intermediate as required by the chemical theories of oxidative phosphorylation already outlined. Some circumstantial evidence exists, however, which indicates that a source of potential energy can be built up in the event of a substrate being oxidised in the absence of Pi and ADP. Reversal of the respiratory chain is considered to be one demonstration of this. As Slater (1964) has pointed out, the nature of this evidence does not permit a firm choice on whether a high-energy intermediate or a membrane potential is the form of accumulated energy.

Reversal of the respiratory chain was first observed by Chance (1956) who noted that succinate added to mitochondria under conditions where phosphorylation was prevented by lack of ADP, reduced the mitochondrial NAD. Later, it was suggested (Chance and Hollunger, 1957) that this was due to reversal of electron flow in the respiratory chain, the necessary energy for the reduction of NAD coming from the oxidation of succinate. These results have been confirmed and extended (Ernster, 1960, 1961; Tager and Slater, 1963). Ernster (1961) for example, showed that in the absence of Pi, succinate oxidation could be linked with the reduction of acetoacetate. Snoswell (1962) observed the reduction of mitochondrial NAD under similar conditions. Furthermore it has been shown that oligomycin is ineffectual in preventing the reversal of electron transport, thereby confirming that the energy required for reversal need not be provided by ATP formed during oxidative phosphorylation, but could be provided by some other source of potential energy formed prior to this (Ernster, 1960; Snoswell, 1961). Other observations made by these authors include the fact that inhibi-

tion of succinate oxidation by Antimycin A or anaerobiosis in accompanied by inhibition of NAD or acetoacetate reduction. Since added ATP can partially relieve this inhibition of NAD reduction in an oligomycin sensitive reaction the conclusion has been drawn that ATP cannot act directly, but only through a postulated high-energy intermediate.

In terms of the chemical theory of oxidative phosphorylation, respiratory inhibition by oligomycin is considered to be a secondary effect induced by the accumulation of high-energy intermediates which are prevented from being phosphorylated. Tager and Slater (1963) have shown that respiration inhibited in this way can be released by the addition of α-ketoglutarate, and conclude that this is due to oxidation of NADH by α-ketoglutarate resulting in a further reduction of the NAD formed, with the concommitant disappearance of accumulated high-energy intermediates. These authors also produced results to indicate a competition between NAD and the phosphorylation step for the postulated high-energy intermediate (Tager and Slater, 1963b). Further support to indicate the utilisations of a high-energy intermediate for energy requiring reactions comes from the work of Van Rossum (1962) in which he showed that added oligomycin could only partially destroy sodium-potassium gradient across the membranes of rat liver cells. Several authors have shown that the uptake of various ions by mitochondria is inhibited (DeLuca and Engstrom, 1961; Brierley, Mura and Green, 1963; Chappell, Greville, Bicknell, 1962) by 2:4-DNP but not by oligomycin. This is in agreement with the thesis that this can be mediated by a high-energy intermediate (A~C) which is hydrolysed by 2:4-DNP but not by oligomycin. At present, therefore, there appears to be substantial agreement among the various schools investigating oxidative phosphorylation, that energy accumulates in mitochondria under conditions when it cannot be converted to ATP.

(5) Isolation of high-energy intermediates:

(5) Isolation of high-energy intermediates:

Recently, several compounds have been described as possible high-energy intermediates of the oxidative phosphorylative reaction (Purvis, 1958, 1960; Suelter, DeLuca, Peter and Boyer, 1961; Pressman and Dallam, 1961; Pinchot, 1963; Webster, Smith and Hansen, 1963). Examination of these shows that at present no single one completely satisfies all the criteria required for such a component. It should be noted, however, that research in this field is at an early stage of development. The most interesting compound appears to be Webster's reduced cytochrome c coupling factor (Webster, 1962, 1963; Webster, Smith and Hansen, 1963) which corresponds in many ways to the postulated A~C intermediate of the chemical theory. This factor has been isolated from sub-mitochondrial particles, and preliminary investigations indicate that it is a high-energy soluble form of cytochrome b formed during its oxidation. It reacts with Pi and ADP to form ATP and cytochrome c in stoicheoimetric amounts. It is significant that this reaction is not inhibited by 2:4-DNP although the formation of the coupling factor is.

Purvis (1958, 1960) has presented evidence for an NAD~C factor on the basis that the sum of the NAD and NADH extractable from liver mitochondria by conventional methods is consistently less than the total NAD present and that the NAD~C intermediate represents the difference. On addition of succinate the extractable pyridine nucleotide decreased, suggesting the formation of more NAD~C on oxidation. Kilingenberg and Bucher (1960) were unable to confirm these findings, but Snoswell (1962) has reported the phenomena in rabbit heart sarcosomes. One of the essential criteria for a high-energy intermediate - that it should be readily phosphorylated to ATP - has not been demonstrated.

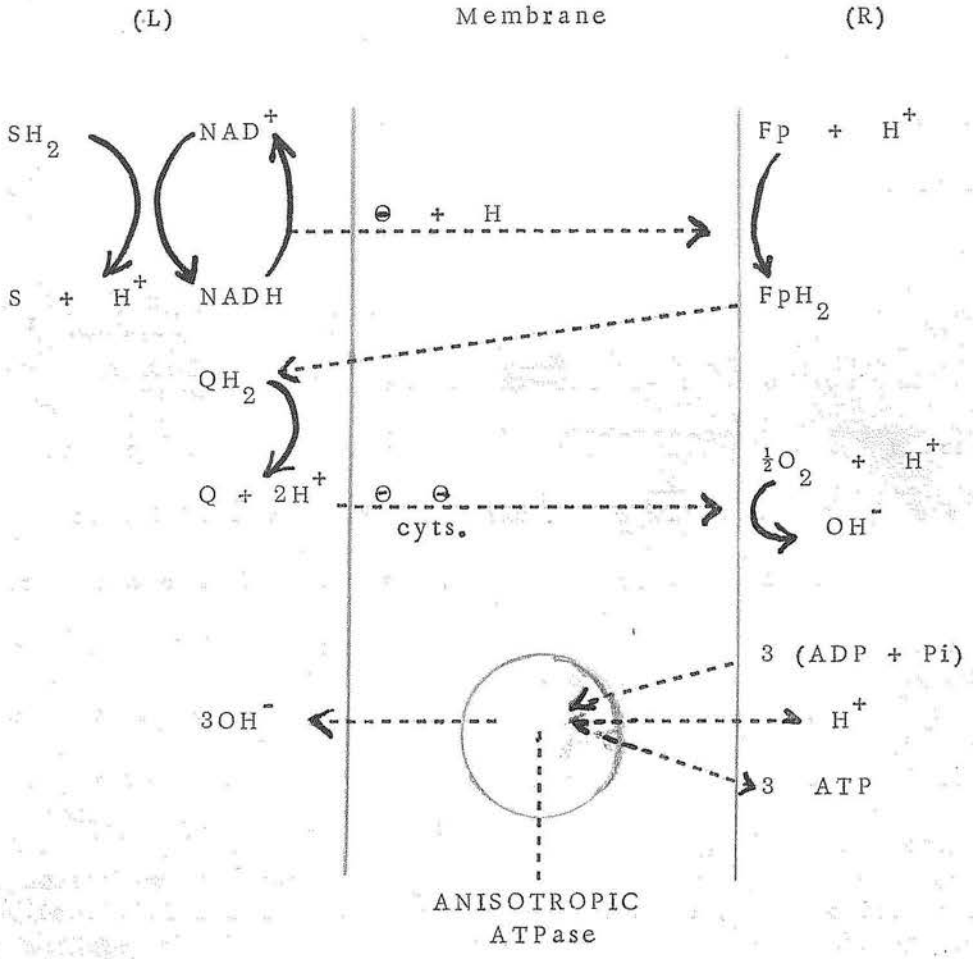
Evidence has been presented by Boyer and colleagues (Suelter, DeLuca, Peter and Boyer, 1961; Peter and Boyer, 1963; Boyer, DeLuca, Ebner, Hultquist and Peter, 1962) that imidazole phosphate participates as an intermediate.

Mitochondria in the absence of ADP rapidly incorporated Pi into this compound, the kinetics and specific activity of the reaction suggesting to the authors a precursor of ATP. Slater, Kemp and Tager (in press) have brought evidence against this on the basis of kinetic and inhibitor studies, and have shown for example, that under their experimental conditions, the rate of incorporation of 32Pi into protein-bound phosphorus was only 1.5% of that into mitochondrial ATP.

B: THE CHEMI-OSMOTIC THEORY

In 1961 Mitchell proposed a chemi-osmotic mechanism of oxidative phosphorylation which provided elegant explanations for certain of the problems inherent in the chemical theories. Mitchell's theory is radically different from the chemical theories, the central feature being that ATP is formed from ADP and Pi by the reverse action of an anisotropic ATPase sited in the mitochondrial membrane in such a way that the H^+ and OH^- ions removed on dehydration of ADP and Pi are discharged on separate sides of the membrane into "sinks" of OH^- and H^+ respectively, created as a result of the specific directional location of the electron carriers. The respiratory chain is thus regarded as a means to bring about a separation of electric charges, delivering H^+ to one side and OH^- to the other side of a charge impermeable membrane, thereby creating an electrochemical activity gradient of these ions across the active centre region of the anisotropic ATPase, which is driven in reverse as a result. The predicted stoicheoimetry of ATP synthesis is one ATP molecule for every electron translocated across the membrane on the basis that the movement of electrons push a corresponding number of OH^- ions from Pi, thus generating phosphorylium for donation to ADP. This is illustrated in the figure overleaf which shows the coupling of phosphorylations to the oxidation of the substrate SH_2 through NAD, flavoproteins (fp), ubiquinone (Q) and the cytochromes (cyts.). The theory requires that the low molecular weight com-

ponents NAD and ubiquinone are freely diffusible in the membrane, whereas the flavoproteins and the cytochromes are not.



Where the suffices R and L indicate the right and left sides of the membrane, the reaction sequence may be represented -

$$SH_2 + NAD^+ \rightleftharpoons S + NADH + H_L^+$$

$$NADH + Fp + H_R^+ \rightleftharpoons NAD^+ + FpH_2$$

$$FpH_2 + Q \rightleftharpoons Fp + QH_2$$

$$QH_2 + 2\,Fe^{+++} \rightleftharpoons Q + 2\,Fe^{++} + 2\,H_L^+$$

$$2\,Fe^{++} + \tfrac{1}{2}O_2 + H_R^+ \rightleftharpoons 2\,Fe^{+++} + OH_R^-$$

$$\text{Sum } SH_2 + \tfrac{1}{2}O_2 + 2H_R^+ \rightleftharpoons S + 3H_L^+ + OH_R^-$$

$$3\,ADP_R + 3\,Pi_R \rightleftharpoons 3\,ATP_R + 3OH_L^- + 3H_R^+$$

$$\text{Sum } SH_2 + \tfrac{1}{2}O_2 + 3\,ADP_R + 3Pi_R \rightleftharpoons S + 3ATP_R + 3\,H_2O_L + H_2O_R$$

The ATPase is regarded as being accessible to ATP, ADP and Pi on the oxygen (R) side of the membrane only, and to OH^- ions on the other side. Synthesis of ATP is depicted as the diffusion of the OH^- ions down a free energy gradient towards the left from inorganic Pi groups which pass to the active centre from the right. The phosphorylium ions (P^+) created by the withdrawal of the OH^- is attacked by a nucleophilic grouping in the active centre, and then donated to the terminal oxygen of ADP-O^- to form ATP.

Mitchell has calculated that if the pH on one side of the membrane is 7.0, a steady state concentration of (OH^-) of about 10^{-14} at pH 7.0 would be required on the other side to promote the synthesis of ATP. As this is highly unlikely, he has suggested that exchange diffusion carriers are present in the membrane allowing strictly coupled one to one exchange of H^+ against K^+ or of OH^- against Cl^- for example, so that the pH differential would be greatly reduced and largely replaced by a membrane potential. Mitchell has produced convincing thermodynamic arguments to support this aspect of his theory.

The most important features of the chemi-osmotic theory relative to the known facts of oxidative phosphorylation may now be listed. It is the first to provide a precise chemical explanation for the experimentally observed P:O ratios of 3 with NAD linked substrates and 2 with succinate. No intermediate phosphorylation steps are involved and only one ATPase system is required. (The unsatisfactory nature of the evidence for high-energy intermediates has already been discussed and more than one dinitrophenol-stimulated ATPase (page 28) has not yet been proved.) Furthermore, it provides an alternative explanation to that of Lehninger (page 14) to account for the close association of oxidative phosphorylation with membranes, noted by many authors (Zeigler, Linnane and Green, 1958; Lehninger, 1959). According to Mitchell's theory, the uncoupling observed in damaged mitochondria merely reflects inability of the membrane to maintain an electrochemical gradient. Although sub-mitochondrial units are capable of carrying out oxidative phosphorylation, Mitchell (1961) has pointed out that coupling in these cases is typically inefficient, and invariably the preparations contain lipid which could well reconstitute a charge impermeable coat around each particle. Lehninger and Wadkins (1962) have observed that the theory can explain aspects of the phenomena of mitochondrial swelling and ion accumulation associated with substrate oxidation (Chappell and Greville, 1960; Packer, 1960; Hunter, 1961). Important experimental evidence to support the chemi-osmotic theory is that uncoupling concentrations of a range of uncouplers catalyse the passage of protons through mitochondrial and bacterial membranes at rates approximately equivalent to the rate of passage of electrons through the respective oxido-reduction systems during uncoupled glutamate oxidation (Mitchell, 1961b). Aspects of this have been confirmed by Greville (1962). This is entirely in agreement with the theory, indicating that the uncoupling brought about by these agents might be due to their

capacity to conduct protons through membranes thereby abolishing the postulated membrane potential which induces ATP synthesis. In this respect Mitchell (1961b) has suggested that the one common feature of the wide range of chemically diverse uncoupling agents is capacity to carry protons, which may be ascribed to the presence of two or more weakly acidic groups between which the proton bonding electrons can pass by way of the m orbitols present in all classical uncouplers. Mitchell's theory can satisfactorily explain the inhibition of respiration in the absence of Pi and ADP on the basis that no OH^- ions can then be formed on the substrate side of the membrane to neutralise the H^+ ions, and that, as the result of exchange of cations for H^+, a high membrane potential is built up which eventually inhibits the passage of electrons. The action of oligomycin in inhibiting respiration can be explained on the same basis, phosphorylation in this case being inhibited by the specific action of oligomycin on the ATPase.

Slater (1964) has enumerated other properties of the oxidative phosphorylation system which are compatible with the chemi-osmotic theory, namely the isotopic pattern of the various exchange reactions already discussed (page 13). It has already been observed (page 16) that although energy apparently accumulates in the absence of phosphorylation, the available evidence does not differentiate between this being in the form of high-energy intermediates as postulated by the chemical theories, or a membrane potential as required by Mitchell's theory. It is clear that the theory can explain the results of Howland (1963) (page 10) with tetramethyl-p-phenylenediamine as substrate, as reduction of cytochrome c might be expected to have the same effect as reduction of cytochrome b, in which a P:O ratio of 2 would be anticipated. Slater (1964) however, has drawn attention to the fact that the selective uncoupling by antimycin of half of the phosphorylations coupled with oxidation of this substrate is difficult to accommodate in the theory. Lehninger and Wadkins (1962)

too, question whether the chemi-osmotic theory in its present form can account for the stoicheoimetry of phosphorylations associated with partial systems such as the NAD --> cyt. c and cyt. c --> oxygen span.

Mitchell's theory is ingenious and attractive and must be considered as a valid alternative to the chemical theories, at least until the involvement of high-energy intermediates in oxidative phosphorylation is unequivocably demonstrated.

THEORIES OF UNCOUPLING

The first experimental evidence that oxidative phosphorylation could be uncoupled by nitrophenols and other compounds came from the work of Loomis and Lipmann (1948) in which they showed that 2:4-DNP effectively inhibited phosphorylations without affecting the respiration of rabbit kidney cyclophorase preparations. Since then uncoupling has been demonstrated by many compounds including salicylates (Smith, 1959), dicoumarol (Martius and Nitz-Litzow, 1953), gramicidin (Hotchkiss, 1946), and various long chain unsaturated fatty acids (Pressman and Lardy, 1952, 1955; Hullsman, Elliot and Slater, 1960). A well established phenomenon which must be accounted for in any theory of uncoupling is that, with a few exceptions such as arsenate (Ter Welle and Slater, 1964), uncouplers induce ATPase activity in isolated mitochondria which do not effectively hydrolyse added ATP in their absence (Kielley and Kielley, 1951; Lardy and Wellman, 1953; Hunter, 1951; Myers and Slater, 1957). Before either uncoupling or stimulation of ATPase activity was demonstrated experimentally, the first hypotheses on the actions of dinitrophenol were proposed by Lardy and Elvejhem (1945), namely that the phenol brought about its effects either by allowing oxidation to proceed in the absence of phosphorylation or by catalysing the hydrolysis of an intermediate phosphate compound. All explanations of the actions of 2:4-DNP since, may

be regarded as modifications or extensions of these hypotheses, with the exception of that of Mitchell (1961) already referred to (page 19).

Lardy himself favoured the second of these hypotheses and proposed that 2:4-DNP catalysed the hydrolysis of a phosphate-containing intermediate by displacing the phosphate from an enzyme complex with the formation of a DNP-complex which then decomposed to liberate the enzyme (Lardy and Wellman, 1953). This scheme is shown below, and is compatible with the observed increase in ATPase activity induced by the uncoupler.

$$\text{Enz} + \text{ATP} \rightleftharpoons \text{Enz.Pi} + \text{ADP} \overset{\text{DNP}}{\rightleftharpoons} \text{Enz.DNP} + \text{Pi} \overset{H_2O}{\rightleftharpoons} \text{Enz.} + \text{DNP}$$

At present this hypothesis appears unacceptable as the weight of evidence is now against the idea that Pi enters the reaction sequence before the point at which uncoupling occurs (page 12).

The theory that 2:4-DNP enables oxidation to proceed without phosphorylation is held by Ernster's school (Low, Siekevitz, Ernster and Lindberg, 1958; Low, 1959; Ernster, 1961), who believe that the energy-rich compound is with the reduced member of the respiratory chain - for example, $BH_2 \sim C$ rather than $A \sim C$ as in Slater's formulation. Ernster's mechanism of the NAD-flavin-linked phosphorylation may be described -

$$\text{NADH} + \text{Fp} + \text{C} \rightleftharpoons \text{NAD}^+ + \text{FpH}\sim\text{C} + \text{OH}^-$$

$$\text{FpH}\sim\text{C} + 2\text{Fe}^{+++} + \text{Pi} \rightleftharpoons \text{Fp} + 2\text{Fe}^{++} + \text{C}\sim\text{P} + 2\text{H}^+$$

$$\text{C}\sim\text{P} + \text{ADP} \rightleftharpoons \text{C} + \text{ATP}$$

$$\text{NADH} + 2\text{Fe}^{+++} + \text{ADP} + \text{Pi} \rightleftharpoons \text{NAD} + 2\text{Fe}^{++} + \text{ATP} + H_2O + \text{H}^+$$

The reaction mechanism of uncoupled oxidation is given as -

$$\text{NADH} + \text{Fp} + \text{H}^+ \xrightarrow{\text{2:4-DNP}} \text{NAD}^+ + \text{FpH}_2$$

and the dinitrophenol-induced ATPase explained by the sequence -

$$ATP + C \rightleftharpoons C\sim P + ADP$$

$$C\sim P + fpH_2 \rightleftharpoons Pi + fpH\sim C$$

$$fpH\sim C + NAD^+ + OH^- \rightleftharpoons fp + C + NADH$$

$$NADH + fp + H^+ \xrightarrow{2:4\text{-DNP}} NAD^+ + fpH_2$$

It will be noted that, according to Ernster's theory, only one reaction is affected by the uncoupler and that this reaction is common both to uncoupled oxidation and the 2:4-DNP induced ATPase activity. The finding by Hemker (1964) that the concentration of 2:6-DNP required to induce maximum ATPase activity is ten times greater than that required for maximum uncoupled respiration is very difficult to reconcile with this theory. Furthermore Hemker (1963) has shown that there is no experimental evidence to support the idea that uncoupling of the third phosphorylation step contributes to the uncoupler-induced ATPase activity.

The theory of uncoupling supported by Slater and co-workers (Slater, 1961; Hemker, 1963) is that the uncoupler reacts with the first high-energy compound to form a labile but not energy-rich nitrophenol compound which readily dissociates. The suggested reaction mechanism of uncoupled oxidation is therefore -

$$AH_2 + B + C \rightleftharpoons A\sim C + BH_2$$

$$A\sim C + DNP \longrightarrow A + C\text{-}DNP$$

$$C\text{-}DNP + H_2O \rightleftharpoons C + DNP$$

$$AH_2 + B \longrightarrow A + BH_2$$

and that for dinitrophenol-induced ATPase -

$$
\begin{array}{lcl}
ATP + C & \rightleftharpoons & C\sim P + ADP \\
C\sim P + A & \rightleftharpoons & Pi + A\sim C \\
A\sim C + DNP & \longrightarrow & A + DNP\text{-}C \\
DNP\text{-}C + H_2O & \rightleftharpoons & DNP + C \\
\hline
ATP + H_2O & \longrightarrow & ADP + Pi
\end{array}
$$

Hemker (1964) and Slater (1964) have argued that this mechanism is not subject to the criticisms applied to Ernster's theory, since there is no reason why the binding of C by DNP should have the same effect on uncoupled oxidation and the ATPase, as no oxidation reaction is included in the dinitrophenol-induced ATPase.

As all three phosphorylations associated with the respiratory chain can be uncoupled, attempts have been made to determine whether there are three separate dinitrophenol-induced ATPases corresponding with the three phosphorylating systems, or only one which is common to all. This work has been inconclusive (Slater, 1961, 1964). Despite initial evidence that three such ATPases were in fact present and could be distinguished by different pH optima (Myers and Slater, 1957), further work by Hemker (1962) showed that this conclusion was unjustified as it was based upon the assumption that the active form of the nitrophenol was the ion and that changes in pH between 5.5 and 10 affected the enzyme system rather than the uncoupler. In fact, Hemker concluded that pK, lipid solubility and pH must be considered in determining the concentration of any one nitrophenol in the lipid phase of the mitochondria. Hemker showed that, typically for any given uncoupling nitrophenol at pH values greater than the pK

$$PC_L = PC_{opt} - pK + pH + pQ$$

where

pC_L = $-\log_{10}$ conc. in mitochondrial lipid

pC_{opt} = $-\log_{10}$ conc. in medium inducing maximum ATPase activity

$pQ = -\log_{10} \frac{\text{conc. undissociated phenol in xylene}}{\text{conc. undissociated phenol in water}}$

The equation is based on the assumptions that the partition coefficient between mitochondrial lipid and water is equal to that between xylene and water, and that the undissociated phenol must be taken up by the lipid before it uncouples.

Although Hemker's results showed that it was not possible to identify more than one dinitrophenol-induced ATPase from the findings of Myers and Slater (1957) the possibility cannot be discounted that there are in fact three dinitrophenol-induced ATPases with rather similar pH optima and DNP sensitivity. Alternatively, one of the three phosphorylative steps may quantitatively dominate the ATPase reaction (Slater, 1961). Hemker (1963) has recently obtained evidence for the existence of two ATPase systems which differ in their sensitivity to Amytal and Antimycin, and the dinitrophenol concentration required to induce maximum activity. His suggestion that the Amytal sensitive system corresponds to the first phosphorylation step and the antimycin sensitive step to the second step in the respiratory chain is consistent with the chemical theory of oxidative phosphorylation, as it is known that these are respiratory inhibitors in the region of these steps (Slater, Colpa-Boonstra and Links, 1961). Conversely, Hemker's findings cannot be easily explained on the basis of Mitchell's theory which postulates that only one ATPase is required - that being independent of the respiratory chain.

It is obvious that resolution of the problem of how many ATPase systems are involved in respiratory chain phosphorylations would represent a major advance towards an understanding of the mechanism of oxidative phosphorylation and its uncoupling.

* * * * * * *

The foregoing outline of current ideas on oxidative phosphorylation derived from studies on sub-cellular systems, is an essential background to the work on intact L strain fibroblasts described in this thesis. Prior to 1948, and their definition as uncoupling agents, virtually all investigations into nitrophenols were carried out on whole animals, isolated tissues and a wide range of unicellular systems. Information from these and later experiments may be briefly summarised under several headings, without comment at this stage on possible explanations for the observed phenomena.

(i) Heat Production:

The earliest observation on the effects of nitrophenols appears to be that of Cazeneuve and Lepine (1885) who noted that administration of dinitro-α-naphthal to dogs induced fever. That uncouplers cause increased heat production during metabolism has been reported by many pharmacologists, and is particularly well documented for the salicylates (Erganian, Forbes and Case, 1947; Barnett, Powers, Benward and Hartmann, 1942). It was shown by Heymans and Bouchaert (1928) and others that this is due to a direct effect of the uncoupler on the tissues, rather than some heat-regulating centre.

(ii) Stimulation of Respiration and Glycolysis:

Nitrophenols have been found to stimulate respiration and glycolysis in many biological systems including frog muscle (Ehrenfest and Ronzoni, 1933), bull sperm (Lardy and Phillips, 1943), guinea pig cerebral cortex (McIlwain and Gore, 1951), certain bacteria (Pickett and Clifton, 1943) and ascites tumour cells (Emmelot and Bos, 1959; Ram, Kalner and Block-Frankenthal, 1963). They have also been reported to stimulate anaerobic glycolysis of frog muscle (Ehrenfest and Ronzani, 1935) and ascites cells (Clowes and Keltch, 1954; Emmelot and Bos, 1959). No direct comparison of the results is possible due

to wide divergences in buffer, pH, residual respiration and other factors from experiment to experiment, and the fact that in most cases the optimum uncoupler concentration for respiration and glycolysis was not determined. Although glucose was the exogenous substrate in such experiments, 2:4-DNP has been shown to stimulate pyruvate and lactate respiration in bull sperm (Lardy and Phillips, 1943) and rat brain cortex (Peiss and Field, 1952). The latter authors noted that this was not the case for succinate and α-ketoglutarate respiration, confirming the findings of Tyler (1950) and Lardy and Phillips (1945) who suggested that this was due to entry problems.

(iii) Ionic Imbalance:

2:4-DNP (0.05mM) and salicylate (0.25mM) have been shown to considerably increase the leakage of potassium from respiring rat diaphragm (Randle and Smith, 1958). Similar observations have been made for rabbit kidney slices (Mudge, 1951). Experiments on human red blood cells have shown that 2:4-DNP (0.05mM) and salicylate (3mM) induce a loss of potassium and gain of sodium and water (Waltner, Csernovszky and Kelemen, 1959).

(iv) Reduced Assimilation:

It has been reported for several biological systems that uncouplers at concentrations which stimulate or do not significantly depress respiration markedly inhibit the assimilation of carbohydrates and amino acids. Much of the work on micro-organisms is difficult to assess precisely as calculations on substrate assimilation have been made on the basis of assumptions about respiration which are far from conclusive (page 103). There is good evidence, however, that dinitrophenols at suitable concentrations induce almost complete oxidation and therefore greatly reduced assimilation, of acetate and butyrate (Clifton, 1937) and lactate, pyruvate and glucose (Doudorouff, 1940) by Pseudomonas species, a wide range of substrates by

Escherichia coli (Clifton and Logan, 1939) and acetate by yeast (Winzler and Baumberger, 1938). The incorporation of 14-C glucose, acetate and pyruvate into isolated rat tissues is substantially inhibited by 2:4-DNP and salicylate (Smith and Moses, 1960; Huggins, Moses and Smith, 1960), as is the incorporation of 14-C amino acids into ascites tumour cells (Rabinovitz, Olsen and Greenberg, 1955; Emmelot and Bos, 1959; Ellis and Scholefield, 1961), the extent of this varying with the metabolism of the cells.

(v) Inhibition of Growth and Division:

As might be anticipated from the foregoing, uncouplers have a general inhibitory effect on growth at concentrations which need not suppress respiration greatly. This has been shown for bacteria (Monod, 1944), budding yeast (Martin and Field, 1934), fission yeast (Faed, 1955) and maize coleoptile (Beevers and French, 1952), and L cells (Cailleau et al, 1956). The last-named paper appears to be the only investigation into uncoupling and tissue culture cell growth, and will be further discussed later.

The effects of nitrophenols on sea urchin eggs have been discussed by Krahl (1950) who concluded that cleavage was inhibited by concentrations which had little effect on respiration. Swann (1955) has shown that 2:4-DNP inhibits cleavage only if it is added before the commencement of mitosis, and has argued that this indicates that the continuous production of ATP is not required to carry division through once it has commenced.

(vi) Necrosis:

At high concentrations, uncouplers are inevitably toxic to biological systems. In this respect dinitrophenols have been widely used as insecticides (Brown, 1951) and herbicides (Blackman, Templeman and Halliday, 1951). The effective toxic concentration varies widely with the uncoupler, the nature of the metabolism of the system and other factors such as pH (Simon, 1953).

Uncoupling agents therefore induce marked changes in metabolism, transport and synthesis consequential to their uncoupling action.

This study is the first phase of an investigation into the extent and nature of these changes in a single cell type, and their relationship to the levels of intracellular adenine nucleotides. The use of an established line of tissue culture cells circumvents some of the difficulties inherent in tissue slice techniques, and provides a system for investigating uncoupling action from several approaches. It employs a single cell type, at least in a superficial sense; Warburg manometry and other techniques can be carried out on single cell suspensions in which every cell is in a microenvironment similar to that of every other cell with regard to pH, aeration and substrate; under conditions used in this laboratory viability remains high during manometric experiments with little cellular breakdown and leakage of metabolites. Furthermore, with the development of methods of mass culture, sufficient cells can be grown to make fractionation and work on sub-cellular systems possible. Possibly the greatest virtue of using tissue culture cells is that essentially short-term manometric experiments can be supplemented by investigations into the cumulative effects of uncoupling over long periods of time.

It should be noted that the status of tissue culture cells is often difficult to define. Established strains have in most cases lost some of the specific characteristics of the tissue of origin and appear to have become adapted or selected for rapid proliferation rather than differentiation (Lieberman and Ove, 1958). An implication of this is the possibility of cell strains becoming less sensitive to humoral control factors than the tissues of origin. It is therefore hazardous to rigidly relate all information from cell strains to the original cell types in vivo. The fact remains that cell strains are highly organised biochemical systems showing all the essential

features of metabolism and synthesis necessary for maintenance and growth. An apologia for the choice of the specific cell type used in these studies follows:

THE STRAIN L FIBROBLAST

Strain L fibroblasts (929) isolated by Earle (1943) from mouse subcutaneous connective tissue and later cloned by Sanford, Earle and Likely (1948) were used in these studies. This strain was used primarily because it was the only cell strain which could be grown as monolayer cultures in polymer-free chemically defined medium (Waymouth, 1959). All other mammalian cells require serum or some other polymer. The disadvantages of the use of serum in nutritional and metabolic studies have been adequately documented (Carrel and Ebeling, 1923; Phillips and Andrews, 1960). For example, reproducibility of results depends to a large degree on the source and condition of the serum used. The fact that in many experiments up to 40% serum has been used indicates that in such cases humoral factors like insulin or thyroid hormones may well be present at sufficient concentration to influence metabolism. Furthermore, the use of serum makes measurements of the uptake and production of metabolites, and chemical analyses of the cells less convenient and reliable. Such difficulties were avoided by the choice of the L cell. In addition it was one of the few cell strains which could be grown en masse in fluid suspension cultures (Danes, 1957; Graff and McCartney, 1957), although all the suspension methods described at the time of commencing this work required media containing serum protein or some other polymer such as methyl cellulose (Bryant, Schilling, Evans and Earle, 1960) to support normal rates of dispersed cell growth. Subsequently a technique was developed in this laboratory (Sinclair, Reid and Mitchell, 1963) which went some way towards

overcoming the problems of cell clumping and necrosis that often accompanies growth in these conditions. (McLimmons et al, 1957; Bryant et al, 1961). This technique involves the use of trypsin in small amounts (10-50ug/l.) in an otherwise protein-free medium. The trypsin appears to function by breaking down an extra-cellular material by which the cells adhere to surfaces and to each other, and it is a characteristic feature of growth in this trypsin-containing medium that the cells mostly exist singly or in pairs.

At best, dispersed cell growth is a fundamental departure from the mechanics of mammalian fibroblast growth in vivo. It has been developed as a means of making large homogeneous cell populations available for manometric and biochemical studies and for this purpose has distinct advantages over monolayer techniques. Even in cases where sufficient cells can be produced in monolayer cultures, the application of Warburg manometric techniques presents some difficulties. The cells must be detached from the glass by either scraping or the use of trypsin or versene - techniques which can damage the cells: metabolism is then measured under conditions of shaking which may be quite foreign to them. No method of dispersed mammalian cell culture is completely satisfactory at present, but within our experience the addition of trypsin to the culture is preferable to the use of serum or methyl cellulose for the present work. Cells grown by this method are conveniently handled and remain in suspension as single cells, with little significant breakdown over some hours when subjected to Warburg manometry. In contrast, cells grown in suspension culture with polymers present tend to show clumping, adhesion to sides and breakdown when shaken in manometer flasks without the polymer present. A point of importance is that the metabolism and growth rate of cells in trypsin-containing media appears to undergo little adaptation to this mode of culture, and if the trypsin is omitted and the cells allowed to settle on glass, they

quickly adhere and grow as monolayers again with a growth pattern similar to other monolayer cultures.

A further reason for using strain L fibroblasts is that a considerable amount of information is already available (Earle, Bryant, Schilling and Evans, 1956; Dales and Fisher, 1959; Maio and Rickenberg, 1962) on the growth and metabolism of this cell type. In extrapolating such information however, the possibility of minor variations between the cells cultured in different laboratories due to continuous selection of variants for different nutritional and physical conditions should be kept in mind (Sanford et al, 1954; Whitfield and Rixon, 1960; Swim and Parker, 1957).

METHODS

A. TISSUE CULTURE FLUIDS

(i) Growth Medium

A culture of NCTC clone 929 (Strain L) fibroblasts in bicarbonate-buffered Eagle's (1955) medium supplemented with 20% horse serum was obtained from Dr. J. McDougall of the Bacteriology Department, University of Edinburgh in August, 1960. For the following three months the cells were grown as monolayers on Medium CSV 6 (Cooper, Wilson & Burt, 1959), a bicarbonate-buffered medium containing lactalbumin hydrolysate and yeast extract as the main sources of amino acids and supplemented with 10% horse serum.

Thereafter, the growth medium used for stock and experimental monolayer cultures was the simple chemically defined medium WB 752/1 (Waymouth, 1959), modified by the addition of 0.0032 mM. ferrous sulphate and 0.25 mM α-ketoglutarate, and an increase in glutamine to 3.4 mM. The modifications were made by Dr. R. Sinclair of this Department on the basis of experience in Waymouth's laboratory and included a reduction in glucose content from 5 per cent w/v to 2.5 per cent w/v. The change in tonicity resulting from this made no apparent difference to cell growth and was not corrected for. The modified medium was bicarbonate buffered to equilibrate at pH 7.3-7.4 with an atmosphere of 5 per cent v/v carbon dioxide in air. The composition of the medium is shown in Table I. Antibiotics penicillin and streptomycin were routinely added at concentrations of 12,500 units and 25 mg. per litre respectively.

Recently a number of chemically defined nutrient media have been described for the growth of the Strain L fibroblast (Evans, Bryant, McQuilkin, Sanford, Westfall & Earle, 1956; McQuilkin, Evans & Earle, 1957; Healey, Fisher & Parker, 1954; Pasieka, Morton & Morgan, 1958). Among the reasons for choosing Waymouth's (1959) medium for the work to be described was the fact that it was relatively

TABLE I

Components of the modified MB 752/1 growth medium

	mg. per litre of MB 752/1	m. molar
SOLUTION Wa: prepared at 5 times the concentrations shown below		
Sodium chloride	6000	103
Potassium chloride	150	2.0
Calcium chloride $2H_2O$	120	0.82
Magnesium chloride $6H_2O$	240	1.18
Magnesium sulphate $7H_2O$	100	0.40
Ferrous sulphate	0.5	0.0032
SOLUTION Wb: prepared at 100 times the concentrations shown below		
Ascorbic acid	17.5	0.1
Glutathione	15.0	0.05
α-ketoglutarate		0.25
Hypoxanthine	25.0	0.18
SOLUTION Wc: prepared at 10 times the concentrations shown below		
L-cysteine HCl	90	0.57
L-lysine HCl	240	1.42
L-arginine HCl	75	0.36
L-histidine HCl	150	0.80
L-glutamic acid	150	1.02
L-threonine	75	0.64
L-valine	65	0.55
L-aspartic acid	60	0.46
glycine	50	0.66
L-proline	50	0.44
L-leucine	50	0.38
L-methionine	50	0.34
L-phenylalanine	50	0.30

TABLE I (contd.)

	mg. per litre of MB 752/1	m. molar
SOLUTION Wc: (contd.)		
L-tyrosine	40	0.22
L-tryptophan	40	0.20
L-isoleucine	25	0.19
L-cystine	15	0.006
SOLUTION Wd: prepared at 200 times the concentrations below		
Choline	250	1.80
Inositol	1	0.006
Thiamine HCl	10	0.03
Nicotinamide	1	0.008
Calcium pantothenate	1	0.002
Riboflavin	1	0.003
Pyridoxine HCl	1	0.003
Cyanocobalamin (B12)	0.1	0.00007
Folic acid	0.4	0.0008
Biotin	0.02	0.00008
SOLUTION Wj: prepared at 50 times the concentrations below		
Disodium hydrogen phosphate	300	2.11
Potassium dihydrogen phosphate	80	0.59
ADDED DIRECTLY:		
Sodium bicarbonate	2240	26.7
Glutamine	500	3.4
Glucose	2500	13.8
Streptomycin sulphate B.P.	25	
Benzyl penicillin	12,500 units	

simple, containing only 40 constituents compared with the 62 of medium CMRL 858 (Healey et al, 1954) and 68 components of NCTC 109 (McQuilkin et al, 1957). Another advantage is that it contains constituent active vitamins rather than the expensive and unstable coenzyme preparations used in other synthetic media. The most satisfactory aspect of the medium was that cells previously grown in high protein biological media as described, proliferated rapidly without any significant retardation during the early passages, and have maintained a constant rate of growth over the years.

Some aspects of the constitution of the medium might be noted. As Waymouth has indicated it contains some amino acids generally regarded as being non-essential (Eagle, 1955). Observations in our laboratory have shown that the omission of L-cystine — L-cysteine being a component of the medium — L-glutamic acid, L-aspartic acid, L-proline and glycine does not significantly affect cell proliferation. The total complement has, however, been retained. Glutamine is present at a relatively high concentration. Extensive utilisation of this amino-acid has been observed before. Paul (1959) and Kitos, Sinclair and Waymouth (1962) have shown that substantial quantities are metabolised to glutamic acid, proline and carbon dioxide. Mohberg and Johnson (1963) found that most of the initial glutamine in their cultures was utilised before cells had reached half of their maximum growth, indicating a role as an energy source for growth even in the presence of glucose. Rapid decomposition rates for glutamine in uninoculated medium have been observed by Tritsch and Moore (1962). Although Eagle, Oyama, Levy, Horton and Fleischman (1956) found that with L cells, non-essential amino acids had a sparing effect on glutamine utilisation, a concentration lower than 3.4 mM was not thought justifiable in the modified Waymouth medium used in the experiments to be described.

The B group vitamins are present in considerably higher concentrations than in most other synthetic media. Recent observations by Mohberg and Johnson (1963)

show that thiamine and ascorbic acid are unstable in tissue culture medium. The authors found that complete omission of ascorbic acid had no effect on growth either in short-term or long-term experiments. The data provided by Waymouth (1959) indicates that in general the vitamin components of the medium have been assessed on a careful experimental basis.

(ii) The Preparation of Growth Medium

Growth medium was prepared from stock solutions shown in Table 1, with the necessary amounts of glucose, glutamine and sodium bicarbonate added before the final volume was attained. Fresh stock solutions were prepared approximately every month, and stored at -20°, in aliquots convenient for the preparation of 1 litre quantities of medium. In preparing the medium, stock solution Wj containing the phosphate buffer salts was added last, after mixing all other stock solutions and ingredients in about 90% of the total volume. This prevented the precipitation of calcium or magnesium phosphates. The medium was sterilised by filtration through Selas 03 filters under negative pressure and dispensed into Universal bottles (20 ml.) and larger (200 ml.) screwcapped bottles. Medium was prepared 2 - 3 times weekly and was used within a few days.

(iii) Other Solutions used in Tissue Culture

Water: All water was distilled through a Scorah all-glass distillation apparatus.

2:4-Dinitrophenol: Due to the limited solubility of 2:4-DNP it was difficult to prepare stock solutions stronger than 10^{-2}M, and as heating to about 70° was necessary even at this concentration, this was undesirable with nutrient medium. Stock solutions were, therefore, prepared at 10^{-2}M concentration in 0.9 per cent sodium chloride and added to growth medium as required. This was acceptable for experiments where the final 2:4-DNP concentration was not much greater than 10^{-4}M, that is a one-hundred-fold dilution of

the sodium chloride. At higher concentrations (10^{-3}M) where the growth medium would be substantially diluted by sodium chloride, 10^{-3}M 2:4-DNP was prepared directly in medium, and other concentrations derived by further dilution with growth medium.

Sodium Salicylate: Sodium salicylate was added directly to growth medium to a concentration of 10^{-2}M. Lower concentrations were obtained by further dilution of this solution by growth medium.

B. TISSUE CULTURE TECHNIQUES

(1) Preparation of Glassware

Before use for tissue culture purposes all glassware was soaked and scrubbed in hot pyroneg solution. After rinsing in hot and distilled water the glassware was dried at 60°C.

Sterilisation of glassware and silicone rubber bungs was achieved by heating at 160°C for one and a half hours. All other apparatus (universal bottles, filters, rubber bungs, etc.) was sterilised by autoclaving for 20 minutes at 15 pounds per sq. inch.

Gassing apparatus: The gas mixture (5% v/v CO_2 in air or nitrogen) was supplied in cylinders by British Oxygen Company Ltd. and passed through 1% aqueous copper sulphate and a sterile cotton wool filter before entering the culture vessels.

(2) Monolayer Cultures

Stock cultures: These were routinely maintained at 37°C in 8 cm. Carrel flasks overlaid by 5 ml. growth medium in equilibration with 5% v/v CO_2 in air. The medium and gas phase was completely renewed every 2 - 3 days and the cells subcultured about once a week.

Experimental cultures: Replicate cultures were seeded in the following manner:- Cells from log phase or early stationary phase cultures were suspended in fresh medium by gentle scraping and pipetting with a pasteur pipette. Medium was added to give a concentration of about 0.15×10^6 cells per ml. Replicate 4.0 ml. quantities were distributed to sterile pre-gassed 50 ml. pyrex conical flasks. The vessels were bunged with Esco white silicone rubber bungs and incubated at 37°C. After 24 hours the medium was removed and experimental medium introduced. After further gassing, incubation was continued. At this point, 4 cultures were sacrificed as initials. The medium was removed and the cells washed by running 5 ml. ice cold 0.9% sodium chloride down

the side of the vessels and over the cells. The saline was then removed by decanting and the flasks inverted for one minute on filter paper. If a cell count was to be made the cells were resuspended in 4 ml isotonic saline by gentle pipetting and 3 samples introduced into Fuchs-Rosenthal chambers, 350 - 450 cells counted in each case. The percentage error of the mean was of the order of 5%. The volume of cell suspension removed for counting was small and constant for each culture. The remainder was spun down (200 g for 10 minutes) and stored in the deep freeze until the end of the experiment.

Where no cell count was required, the washed cells were stored as monolayers in the culture vessels in the deep freeze ($-20^{\circ}C$).

During experiments replicate cultures (usually four) for each set of conditions were sacrificed at intervals and dealt with in a similar manner to the initials. The washing procedure outlined above is however only satisfactory for cells adhering well to the floor of the vessel. Where cultures were necrotic, cells were found in the medium and the saline wash. These were spun down and estimated with those in the flasks.

(3) Roller Bottle Culture

Roller bottle cultures provide a high cell:medium ratio and a means of measuring the rate of uptake of substrates over relatively short periods of time. Typically 500 ml. pyrex bottles (diameter 8 cm.) received approximately 6×10^6 cells in 20 ml. growth medium, and were rotated on a roller drum at 8 revolutions per hour. The medium and gas phase were renewed every 2 - 3 days and within a week a complete monolayer of $30 - 35 \times 10^6$ cells covered the glass. The medium was then removed and glucose-free medium rotated over the cells to remove glucose containing medium held in the intracellular spaces. Experimental medium was then introduced (15 ml. in most cases). During experiments over 24 hours, five 1.5 ml. quantities were removed at

various intervals and stored in the deep freeze for chemical analysis. At the end of experiments the medium was removed and 10 ml. of cold isotonic saline used to wash each monolayer. Cells were counted after suspension in 20 ml. saline and further dilution, or stored as monolayers at -20°C until chemically analysed.

(4) Suspension cultures

Cells were grown in suspension according to the method of Sinclair, Reid and Mitchell (1963). This is a conventional spinner technique using a silicone rubber covered follower impelled at 200 r.p.m. by a magnet attached to a clock motor. The cells were grown in a 10 litre long-necked, flat-bottomed flask in the modified Waymouth medium MB 752/1 supplemented with small quantities of crystalline trypsin (50 ug. per litre on addition). Fresh medium was filtered directly into a refrigerated reservoir and then pumped into the culture vessel through a silicone tube and a drawn out capillary pipette. A Watson-Marlow metering pump controlled by a time clock was used for this. Feeding was semi-continuous, and adjusted so that the starting volume of cell suspension (usually 0.5 litre) was trebled after 4 days when two volumes were siphoned off. The system is stable, cells remaining in exponential growth at between 0.4 and 0.7 x 10^6 per ml for more than two years. The risk of bacterial infection has been minimised by fitting culture vessels with gas and medium ports which make it unnecessary to open vessels. During growth, the gas phase was changed every day.

From time to time, cells have been used for manometric experiments from batch fed exponentially growing suspension cultures as well as the semi-continuous system. Batch fed cultures were grown under the same physical conditions outlined above and received one-third fresh medium daily, followed by 0.1 ml 0.001 per cent trypsin in balanced salt solution per 100 ml. cell suspension. No obvious metabolic differences have been observed in cells from the two systems.

(5) Growth under Anaerobic Conditions

With the use of a manifold, sterile flasks were gassed with 5% v/v CO_2 in oxygen-free nitrogen for 20 minutes and firmly bunged. Any antimetabolites to be used such as 2:4-DNP were present in the flasks during the gassing. A roller bottle containing the cells to be used for seeding the experimental cultures was then gassed for 15 minutes after which the medium was removed and medium which had been subjected to gassing for 30 minutes added. The cells were suspended by scraping and pipetting and during this operation gassing continued. With technical assistance aliquots were dispensed to the culture vessels. Each was then gassed for a further 10 minutes.

(6) Measurement of pH

A Pye pH meter was used to measure the pH of tissue culture fluid. To enable convenient measurement of small quantities in the growth vessels, the glass electrode was enclosed by a tightly fitting perspex shield with windows, and the calomel electrode was extended into a thin potassium chloride bridge. As the pH of bicarbonate buffered MB 752/1 rose rapidly on exposure to air, the pH was measured within a few seconds of removing the bung from each vessel.

Titration techniques:

(i) Maintenance of pH by addition of alkali

In certain experiments (page 87) the pH fall brought about by 2:4-DNP induced glycolysis was checked by alkali titration. At intervals of 24, 48, 60, 72 and in most cases 84 hours, the cultures were titrated to a pH 7.4 standard colour by addition of 0.2 N NaOH from a 0.1 ml. graduated pipette. Before titrating at 24, 48 and 72 hours, three or four replicate cultures were sacrificed, their pH measured and the media and cells retained for analysis. Two cultures were typically sacrificed before titration at 60 and 84 hours.

In this way, graphs (graph 5) were prepared showing the lowest pH values of the cultures during the experiment.

Any changes in tonicity or nutrient concentration caused by titration were very small as about 0.2 ml. alkali was added over the course of experiments to an initial volume of 4 ml. Nevertheless to control for this, and any changes in the gas phase caused by removing the bung, equivalent quantities of isotonic saline were added to all other cultures. Changes in volume were taken into account when calculating glucose uptake and acid production.

(ii) Lowering of pH by addition of acid

In some experiments, the fall in pH induced by 2:4-DNP was simulated in cultures without the uncoupler by periodic addition of acid. A graph was prepared of the fall in pH with time in 0.1mM 2:4-DNP from the results of prior experiments. The quantities of 0.2 N HCl required to be added to cultures growing in the absence of uncoupler, to simulate such a pattern were experimentally derived. In experimental cultures, acid was titrated from a 0.1 ml pipette at intervals of 10, 36, 48, 60, 72 and 86 hours. Because of the problem of handling large numbers of replicate cultures, cultures were sacrificed for pH measurement and analysis at 24, 48, 60, 72, 86 and 96 hours only. As with alkali titration, the dilution of culture media by the acid was small, but equivalent quantities of isotonic saline were added to the control cultures, and account taken of this for medium analysis.

C. MANOMETRIC METHODS

(1) Manometric Medium

The medium used was a modified version of Krebs Ringer Phosphate (KRP) and contained:

sodium chloride	110mM
potassium chloride	4.1mM
calcium chloride	0.4mM
magnesium sulphate	0.5mM
disodium hydrogen phosphate	25.0mM

The medium was freshly prepared for each experiment from five times isotonic salt solutions stored at 4°C. The phosphate solution was adjusted to pH 7.4 with I.O N hydrochloric acid and the pH of the final medium was always checked before use. The medium differs from standard Krebs Ringer Phosphate in that it has a greatly increased buffering capacity (25mM phosphate compared with 15mM). To prevent precipitation of phosphate as the calcium or magnesium salt, it has been necessary to reduce the calcium and magnesium in Krebs Ringer by 80% and 50% respectively. Those changes imposed no significant differences in respiration rates or initial rates of glycolysis.

Substrates: For all substrates other than succinate, an isotonic solution was prepared in distilled water, and thereafter diluted with KRP to give a solution containing the substrate at 10X the concentration required. As the final volume of fluid in the manometer flasks was 3.0 ml, 0.3 ml of the substrate solution was introduced to a sidebulb of each flask. Control flasks received 0.3 ml of the appropriate solution of KRP + isotonic saline.

Uncoupling Agents: 2:4-Dinitrophenol was used at concentrations sufficiently small to neglect its effect on the tonicity of the medium. Due to its low solubility, stock solutions of 10mM were prepared in KRP. Further dilution by KRP was carried out to give solutions 10X the concentrations required. These solutions were added to same sidebulbs as the substrate in 0.3 ml. volumes. Control flasks received 0.3 ml of KRP.

Sodium salicylate was used at much larger concentrations than 2:4-dinitro-

phenol and its effect on tonicity had to be considered. The uncoupler was therefore dissolved in distilled water to 150mM which was 15X greater than the greatest concentration of salicylate required in the final medium. Any further dilutions of this were made with isotonic sodium chloride. 0.2 ml volumes of 15X final concentration salicylate were dispensed to the sidebulbs, plus 0.1 ml of KRP. It will be noted therefore that the addition of salicylate imposed a 6.7% reduction in the phosphate content of the final medium, irrespective of the concentration used. For control flasks, 0.2 ml isotonic saline and 0.1 ml KRP were dispensed to the sidebulbs.

(2) Manometric technique

Cells growing exponentially in suspension were washed once in Krebs Ringer Phosphate (pH 7.4) and resuspended to a concentration of approximately 8 million cells per ml. in Krebs Ringer Phosphate in a conical flask of appropriate size (usually 200 ml.). The cells were then reciprocated (stroke length 4 cms.) at 80 strokes per minute for 1½ hours at 37°C. This pre-incubation period was essential to reduce the endogenous respiration rate from an initial of 17 - 21 ultrs. oxygen per mg. cell protein hour to 10 - 13, a rate which maintained itself for a further 3 hours or more. After preincubation the cells were spun down and resuspended in Krebs Ringer Phosphate to approximately 10 million cells per ml. Samples were removed for counting and protein assay, and 2.4 ml. aliquots were quickly dispensed to 25 ml. Warburg manometric flasks, the sidebulbs of which already contained the substrate, uncoupler, or control medium for these, to a volume of 0.6 ml.

The centre well of every flask contained a filter paper saturated with 0.2 ml. of 2N potassium hydroxide presenting a surface area of about 1 cm^2 to the atmosphere. Adequate aeration was achieved by reciprocating at 80 cycles per minute. Standard manometric techniques were observed. Except where otherwise stated, substrate and uncoupler were added to the main compartment

together about 20 minutes after the taps were closed. Readings were taken every five or ten minutes. Unless the cells were required intact at the end of the experiment for estimating necrosis or 14-C uptake, experiments were terminated by acid tip (0.2 ml. of $1NH_2SO_4$). The contents of each flask were quickly spun down at 1200 g. for 10 minutes and the supernatants, and on some occasions, the residues, retained for analysis.

Anaerobic conditions: Flasks prepared as described were nitrogen gassed for 30 minutes while reciprocating at 80 cycles per minute. Care was taken to flush the dead space in the manometers during this procedure. After closing the taps the flasks were allowed to equilibrate for a further 15 minutes before tipping. As considerable anaerobic endogenous glycolysis occurred during the gassing period, flasks were sacrificed at this point to give a measurement of this. In most anaerobic experiments, metabolism was terminated by an acid tip. In some cases it was essential to estimate the fall in pH during the experiment. The procedure followed then was to rapidly transfer the flask contents to ice cold centrifuge tubes and spin off the cells at 200 g. for 5 minutes at $0^{o}C$. The pH of the medium was then measured at room temperature. A similar procedure was necessary for measurements of 14-C AIB uptake (page 70). In such cases technical help was essential to keep the time to a minimum.

Experiments at different pH values: In such experiments, the cells were always preincubated as described in Krebs Ringer Phosphate, pH 7.4. Aliquots were then dispensed to centrifuge tubes, spun down and the supernatants discarded. The cells in each tube were resuspended in Krebs Ringer Phosphate (adjusted to the appropriate pH by addition of 0.2N HCl or 0.2N NaOH) to a concentration of about 10×10^{6} cells per ml. Aliquots (2.4 ml) were then dispensed to manometer flasks. The contents of sidebulbs were in all cases adjusted to the appropriate pH.

Succinate Experiments: In certain experiments with succinate the substrate was present at very high concentration (42.5mM). The usual manometric technique was altered to accommodate this, by reducing the volume of the cell suspension in the main compartment from 2.4 mls to 2.0 mls, and increasing the volume in the sidebulbs from 0.6 ml to 1.0 ml. For the highest succinate and 2:4-DNP concentrations in the final volume (42.5mM and 1mM respectively), the sidebulbs contained 127.5mM sodium succinate and 3mM uncoupler in distilled water. Lower concentrations were obtained by substituting isotonic sodium chloride (154mM) for sodium succinate as required, and adjusting the concentration of uncoupler.

Cell stains: It was important to obtain an approximate estimate of cell necrosis induced by conditions such as uncoupling. The difficulties of characterising cell necrosis in its early stages are well known (Reid, 1960). Two criteria were considered in respect of this - damage to the plasma membrane and damage to the respiratory mechanism of the cell.

The first of these was examined using a sulphonated dichloro-s-triazinyl azo dye found by Maddy in this laboratory, to be unable to permeate intact plasma membranes. This dye (M.W.643) was prepared 1% in isotonic saline and 1 volume added to 4 volumes cell suspension in a test tube. After mixing, samples were introduced into a Fuchs-Rosenthal counting chamber. Necrotic cells stained a distinctive red colour in contrast to other cells which showed a scarcely discernible pink. The percentage of stained cells did not increase significantly over 30 minutes. To facilitate rapid counting, cell suspensions from manometric experiments were diluted to about 10^6 cells per ml. with KRP. About 600 cells were counted in each case, using two tally counters, one for total cells and one for stained cells. The percentage error was of the order of 10%. With technical assistance, six samples could be counted in under 30 minutes. Nevertheless speed remained a problem which was not resolved

satisfactorily when more than six samples required counting. In such cases, about 300 cells were scanned per sample in a definite order which was reversed for a further count of 300 each. The final proportion was taken as the mean of both counts.

On several occasions oxidative ability of cells subjected to uncoupling was examined to indicate the extent of necrosis. Reduction of the tetrazolium salt - 2,3,5 triphenyl tetrazolium chloride - was used to assess this (Smith and Lester, 1960; Novikoff, 1961). The tetrazolium salt did not accept electrons in the presence of oxygen, and 1 volume 0.8% tetrazolium in isotonic saline plus 3 volumes cell suspension were placed in Thunberg tubes and evacuated before incubation. After 30 minutes incubation at 37°C the cells were scanned microscopically. The proportion of cells showing red granules in the cytoplasm was estimated from a total count of about 600 cells.

Cells damaged by freeze-thawing: After preincubation, thick suspensions of cells in Krebs Ringer Phosphate (7.4) were quickly frozen by deep freezing and kept in this state for 5 minutes before being allowed to thaw at room temperature. This procedure was repeated twice, after which Krebs Ringer was added to bring the suspension to a concentration of approximately the same order as that used for intact cells. Aliquots were quickly dispensed to manometric flasks.

Mitochondrial preparation and incubation: The procedure for the preparation of mitochondria and the measurement of their respiration was slightly modified from that of Slater and Myers (1957). Thick cell suspensions in 5 mls ice cold 0.25M sucrose were subjected to the action of a tightly fitting glass pestle rotating in a 50 ml. polypropylene centrifuge tube at 0°C (approximately 1000 r.p.m. for 3 minutes). The volume was then increased to 20 mls by addition of sucrose. Nuclei and any unbroken cells were spun down at 1000 g. for 5 minutes, washed in ice cold sucrose and the procedure repeated. The

combined supernatants were spun at 6000 g for 40 minutes, the temperature being maintained at $0^{o}C$ during this time. The volume of the residue was small relative to that of the supernatant and without further washing, was suspended in sucrose, and dispensed in 0.5 ml. aliquots to manometric flasks containing the medium used by Slater and Myers (1957). The final volume of fluid in each flask was 4.0 ml., and each contained mitochondria from cells equivalent to about 20mg. protein. Incubation of mitochondria was carried out at $30^{o}C$ and 80 cycles per minute.

Medium for mitochondrial incubation - (after Slater and Myers, 1957).

Main compartment	Mitochondrial suspension	0.5 ml.
	0.6M. Tris/Cl. pH 7.4	0.4
	0.1M. Mg. Cl_2	0.2
	4.0M. KCl	0.1
	0.1M. sodium versenate	0.08
	Water	2.32
		3.6
Side bulb	Sodium succinate	0.4
	TOTAL VOLUME	4.0 ml.

D. THE ANALYSIS OF THE MEDIUM

(1) The Estimation of Glucose

Anthrone Estimation of Glucose

The Anthrone method for the estimation of glucose was first used by Dreywood (1946) and applied by Trevelyan and Harrison (1952) to the study of yeast metabolism. It has been widely used in analyses of tissue culture medium and estimates from 10 ug to 100 ug glucose per ml. Interfering substances (e.g. tryptophane) must first be precipitated from the medium using barium hydroxide and zinc sulphate.

Reagents: 0.30 N barium hydroxide
0.30 M zinc sulphate
0.2% anthrone in 70% sulphuric acid (prepared freshly for each series of estimations).

Procedure: One ml samples of the media to be estimated were diluted with distilled water to give glucose concentrations of between 100 ug. to 1000 ug. per ml. One ml samples of the diluted media were placed in test tubes and diluted with a further 2.0 ml water. To each tube was added 0.5 ml. 0.3N barium hydroxide, and 0.5 ml. 0.3M zinc sulphate. After mixing and standing for 10 minutes, the resulting precipitate was centrifuged at 1200 g for 5 minutes, and washed with 3 ml. and 2 ml. portions of water. The supernatant and washings were made up to 10 ml with water. Triplicate samples of 1.0 ml each were placed in test tubes in a water bath at room temperature, and 5.0 mls Anthrone reagent added to each from a fast-flowing burette. Each solution was thoroughly mixed immediately after the addition of the anthrone. The tubes were then placed in a boiling water bath for exactly 10 minutes. After cooling the green coloration was read at 625 mu using 1 cm. cuvettes. Reagent blanks and standard glucose samples (54 ug. per ml.) were estimated with each series, and the unknown amounts of glucose calculated. The Percentage error of the method is under 2%.

Enzymic Estimation of Glucose

This method has been developed from that published by Salomon and Johnson (1959) to measure from 0.2 to 2.0 u moles (36 to 360 ug) glucose contained in a 2 ml. sample. The method is specific for glucose and protein free media can be used without prior precipitation of tryptophane, etc. It has not been used however in experiments where 2:4-DNP has been present in the media as this causes considerable interference.

Reagent: 100 ml Acetate buffer, prepared by mixing 50 ml. 1.5M acetic acid and 50 ml. 1.5M sodium acetate, and containing 0.240 gm. o-toluidine, HCl, 10 mg. purified glucose oxidase and 5 mg. peroxidase. This reagent was stored in a dark bottle and was stable for 6-8 weeks.

Procedure: The media to be estimated was diluted with water to give glucose concentrations of 0.1 to 1.0 u moles per ml. Duplicate 2 ml. samples were pipetted into test tubes and 1.5 ml reagent added to each. After mixing the tubes were placed in a water bath at 37° C for exactly 30 minutes, cooled and read at 365 mu or 635 mu. Reagent blanks and standard glucose samples (0.6 u moles contained in 2 ml.) were estimated with each series of experiments and the unknown amounts of glucose calculated.

(2) The Estimation of Lactic Acid

The method used was that developed by Hullin and Noble (1953) from the Barker and Summerson (1941) procedure. Lactic acid is converted to acetaldehyde by warm concentrated sulphuric acid containing a catalytic amount of copper. The violet colour produced by the reaction of para-hydroxy diphenyl with acetaldehyde is then used to give a colorimetric estimate of the lactic acid present. The range of the method is up to 0.1 u mole lactate (9.0 ug.) in a 1.0 ml sample. An initial copper lime precipitation is necessary to remove excessive amounts of pyruvate and interfering amino acids.

Reagents: 20% Copper sulphate 5 H_2O
Solid calcium hydroxide
5% copper sulphate 5 H_2O
Concentrated sulphuric acid
1.5% 4-hydroxydiphenyl, prepared by dissolving 1.5 gm in 10 ml. 5% NaOH and diluting to 100 ml. with dist. H_2O.

Procedure: An initial dilution of the protein free media was made with distilled water, to bring the lactic acid content to within the range 0.1 to 1.0 u moles per ml. One ml. samples were placed in test tubes and each received 1.0 ml. 20% copper sulphate and 6.0 ml dist. water, followed by 1.0 gm solid calcium hydroxide. The solutions were mixed vigorously and allowed to stand for 10 minutes. After centrifuging (2,000 rpm for 10 minutes) the supernatants were removed to graduated vessels and the precipitates washed with 2.0 ml and 1.0 ml distilled water. Washings were added to the supernatants and each made up to 10.0 ml. If excessive amounts of pyruvate were present (e.g. ten times the lactate conc.) the copper lime precipitation was repeated twice more as follows: To 8.0 ml. samples, 0.6 ml 20% copper sulphate and 0.6 gm calcium hydroxide were added, and the ppts washed with 2.5 ml dist. water. The supernatants and washings were made up to 11 ml. 0.3 ml copper sulphate and 0.3 gm calcium hydroxide were added to 5.0 ml samples taken from the 11.0 ml solutions, and the ppts washed with 1.5 ml dist. water. The supernatant and washing was made up to 6.5 ml in each case. Results were calculated on the basis that in each case no lactic acid was left in the ppts after washing. Test series with and without added pyruvate indicated that this procedure was satisfactory.

Triplicate 1.0 ml. samples together with reagent blanks and standards (0.05 u mole lithium lactate per ml.) were pipetted into test tubes, and one drop of 5% copper sulphate, $5H_2O$ added to each tube. All tubes were placed in an ice bath and six mls conc. sulphuric acid added to each, dropwise from

a burette. Individual tubes were shaken vigorously in a small ice bath during this addition to prevent rise in temperature, then immediately stoppered and returned to the larger bath. The stoppered tubes were heated at 60° for 30 minutes and returned to the ice bath. When cold, the stoppers were removed and 0.1 ml. 1.5% 4-hydroxydiphenyl added and mixed well. The tubes were heated for a further 20 minutes at 30° to develop the colour. Excess reagent was broken down by heating in a boiling bath for 2 minutes. On cooling, the colour density was read at 560 mu in 1 cm. cuvettes, and the unknown amounts of lactic acid calculated.

(3) The Estimation of α-keto Acids

The method used was that generally employed to measure the α-keto acid content of blood (Friedmann and Haugen, 1943; Cavallini, Frontali and Toschi, 1949). It depends upon the formation of the phenylhydrazone derivatives of the α-keto acids. These are extracted in chloroform and measured spectrophotometrically as a red colouration in alkaline solution.

Reagents: 0.1% 2:4-dinitrophenylhydrazone in 2N Hydrochloric acid
Chloroform (analar)
1.5N sodium hydroxide

Procedure: Initial dilutions with distilled water were carried out where necessary to reduce the α-keto acid content of the media to be analysed to under 0.20 u moles per ml. One ml. samples were transferred to test tubes and diluted with a further three ml. water. One ml. 0.1% dinitrophenyl-hydrazone in 2N HCl was added to each and the tubes were allowed to stand for fifteen minutes at room temperature. The phenylhydrazones formed were removed by extracting three times with 2.5 ml portions of chloroform. 4.0 ml. 0.1N NaOH were added to the chloroform extract, and after mixing and allowing to settle, 3 mls. of the alkaline layer containing the red colour of the derivatives were removed. 1.5 mls of 1.5N sodium hydroxide were

added to each tube to stabilise the colour. Reagent blanks and standard pyruvate samples (0.15 u moles per ml.) were estimated with each series and the amount of α-keto acid in each sample calculated. The percentage error of the method was 5%.

(E). THE ANALYSIS OF TISSUE

(1) Chemical Estimations

Cells were dissolved in 0.20N sodium hydroxide to give a concentration of 100 - 400 ug. protein per ml. (approximately 0.3 - 1.2 x 10^6 cells per ml.). Measurements of protein, total ribose and deoxyribose were made on such alkaline digests as follows:-

PROTEIN : An aliquot of the digest was diluted by an equal volume of distilled water. Triplicate 1 ml. samples of this 0.1N sodium hydroxide solution were estimated for protein by the method of Lowry, Rosebrough, Farr and Randall (1951).

Reagents:
- 0.20 N sodium hydroxide
- 4% sodium carbonate
- 2% sodium potassium tartrate
- 1% copper sulphate
- Folin-Ciocalteu reagent.

Copper alkali solution was freshly prepared by mixing 100 mls 0.20N sodium hydroxide, 100 mls 4% sodium carbonate, 2 mls sodium potassium tartrate and 2 mls 1% copper sulphate in that order. Commercial Folin-Ciocalteu reagent (BDH) was diluted 3-fold with distilled water to make it 1.0 N acid.

Procedure: 5 mls copper alkali solution were added to each protein sample, mixed and left at room temperature for 10 minutes. Diluted Folin-Ciocalteu reagent (0.5 ml) was added as rapidly as possible and mixed immediately. The tubes were placed in the dark for 30 minutes to allow the colour to develop and read at 740 mu against a water blank. The protein standard used was 120 ug bovine serum albumen (crystalline) per ml. The percentage error was 2%.

RIBOSE and DEOXYRIBOSE: Before estimating these, the protein in the cell digest was precipitated. Perchloric acid (6N) was added to the 0.20N digest in a ratio of 1 part to 8 parts. The tubes were heated at 70°C for 15 minutes, cooled and centrifuged (1200 g for 5 minutes). Ribose and Deoxyribose were then estimated in the supernatant by the following methods:-

RIBOSE: The method of Schneider (1957) for the estimation of ribose by the orcinol reaction was used. This method is suitable for estimating 2.5 to 25 ug ribose.

Reagents 1% orcinol in 0.5% $FeCl_3$ in concentrated hydrochloric acid.

Procedure: 1 ml samples of the alkaline cell digest each received 1 ml of distilled water, followed by 2 mls of reagent (freshly prepared). The tubes were boiled for 20 minutes, cooled, and the characteristic green colour read at 660 mu. Water blanks, and standards containing 15 ug ribose were estimated. The percentage error was 3%

DEOXYRIBOSE: The Diphenylamine method for the estimation of Deoxyribose was used (Burton, 1956). This method is suitable for amounts of DNA containing 0.02 to 0.25 ug. atom DNAP per ml.

Reagents 1.5% diphenylamine in 1.5% sulphuric acid in glacial acetic acid. Just before use 0.5 ml aqueous acetaldehyde (16 my/ml) is added to this mixture. This reagent is unstable.

Procedure: To 1 ml samples of the supernatant, 2 mls reagent were added and mixed. The tubes were incubated at 25 - 30° for 16 hours and read at 600 mu. Blanks of 0.5N perchloric acid and standard deoxyadenosine (0.10 u mole per ml in 0.5N PCA) were treated similarly.

ACID-SOLUBLE RIBOSE: In certain experiments (Section 2) it was of interest to estimate the ribose in the acid-soluble cell fraction. Although trichloroacetic acid is widely used for extraction of low molecular weight compounds

(Hutchison & Munro, 1961), it is known to interfere with the Orcinol reaction (Ceriotti, 1955). Perchloric acid was found to be suitable if it was used at 0.2N concentration and the temperature was kept at $0^{\circ}C$. Under these conditions no significant loss of RNA occurred within 45 minutes. The following procedure was adopted:-

Cells were removed from duplicate experimental cultures by scraping and pooled in 10 ml centrifuge tubes. After centrifugation, the media was removed and the cells washed twice with Krebs Ringer Phosphate (5 ml). The tubes were placed in an ice bath and 1.0 ml 0.2N PCA added to each. The cell pads were broken up with a glass needle and the tubes centrifuged at $0^{\circ}C$. (2000 r.p.m. for 5 minutes). The procedure was repeated and the supernatants combined. The PCA extractions took an average of 20 minutes to complete. Ribose content was measured by the Orcinol reaction. For small quantities of tissue the volume of the supernatant was reduced by evacuation to increase the concentration of ribose to a measurable amount. After extraction, the residue was dissolved in 0.2N NaOH as for protein, and protein, ribose and deoxyribose estimated in this fraction as described.

(2) Enzymic Estimations

LACTIC DEHYDROGENASE AND MALIC DEHYDROGENASE: The activities of LDH and MDH were measured on the principle that the oxidation of NADH observed by spectral change at 340 mu is a direct measure of the reduction of pyruvate to lactate in the case of LDH (Kornberg, 1955) and oxaloacetate to malate in the case of MDH (Ochoa, 1955). The activities were in fact very similar and were measured on aliquots of the same cell preparation.

Preparation of crude homogenate: Cells from experimental cultures were washed once, resuspended in Krebs Ringer Phosphate and counted. The cells were then spun down in 10 ml centrifuge tubes and the supernatant discarded. The tubes were put in an ice bath and ice cold distilled water added to a

calculated concentration of 0.45×10^6 cells per ml. The suspensions were kept ice cold for 15 minutes and periodically mixed with glass needles. Thereafter 2 one-ml aliquots were removed for protein assay. The remainder was diluted 1:1 with ice cold distilled water to bring the protein content to approximately 85 ug per ml. This was kept ice cold and used for enzyme assays as follows:

LACTIC DEHYDROGENASE:

Reagents: Sodium pyruvate 0.01M
NADH (0.002M)
KH_2PO_4 - K_2HPO_4 buffer (0.1M pH 7.4)
Sodium chloride (0.01M)
Enzyme solution.

Procedure: The reagents were pipetted into a cuvette of 1 cm. light path and 3 ml capacity - 0.1 ml sodium pyruvate, 1.0 ml phosphate buffer, 0.2 ml sodium chloride, 0.2 ml enzyme solution (containing approximately 17 ug protein) and 1.4 ml distilled water. The cuvette was placed in position in the Unicam SP500 spectrophotometer which was set to 340 mu and an optical density of approximately 0.4. The reaction was started by blowing 0.1 ml of NADH through a 0.2 ml blow out pipette which had its point immersed in the reagents in the cuvette. The pipette was withdrawn from the liquid before the last drop was expelled. This procedure was found to be the most satisfactory for rapidly mixing the NADH with the enzyme and reagents. The first reading was taken 15 seconds after the insertion of the pipette, and thereafter at 15 second intervals for 3 minutes.

The amount of cell protein added was that which had been established as causing a decrease in optical density of between 0.02 and 0.05 per minute. The decrease was measured between 30 seconds and 75 seconds, after which the rate decreased significantly. Readings were taken against a blank containing all reagents except the NADH. The oxidation of NADH by the enzyme

preparation in the absence of pyruvate was negligible.

Unit: A unit of enzyme activity was taken as the amount which causes a decrease in O.D. of 0.001 per minute.

Specific activity: Units of enzyme activity per mg. protein.

MALIC DEHYDROGENASE: Measurement of activity was precisely as for LDH, except that 0.0074 M oxaloacetate was added instead of pyruvate. As recommended in the method of Ochoa (1955), the oxaloacetate was freshly prepared for each assay. Enzyme units and specific activity were as for LDH, and in this case too, no activity was found in the absence of substrate.

ALDOLASE: Preparation of crude homogenate: Cells in experimental cultures were washed once by running isotonic sodium chloride over the monolayers, and then suspended in isotonic sodium chloride to a concentration of about 10^6 cells per ml. After counting, the cells were spun down in 10 ml centrifuge tubes and the supernatant discarded. The tubes were placed in an ice bath and ice cold distilled water added to give a concentration of about 0.5×10^6 cells per ml. The suspensions were kept ice cold for 15 minutes and periodically mixed with glass needles. Thereafter two 1 ml aliquots were removed for protein assay and the remainder used directly for enzyme assay. This procedure was adapted to ensure that the crude homogenate contained between 100 and 200 ug. protein per ml, quantities which were established as having activities directly proportional to the colour intensity developed by the method to be described.

N.B. Aldolase activity was very quickly lost even when homogenates were kept at $0^{\circ}C$. All estimations were made within 2 hours of breaking the cells.

Principle: Aldolase activity was estimated by the method of Sibley and Lehninger (1949). The principle of the method is that fructose-1:6-diphosphate is incubated with the buffered test sample and hydrazine which fixes the triose phosphates formed. The reaction is stopped with trichloroacetic acid which precipitates the protein. The filtrate is treated with alkali followed by acid 2:4-dinitrophenolhydrazine. On making the mixture alkaline again, a characteristic colour appears with maximum absorption at 540 mu owing to the formation of a 2:4-dinitrophenolhydrazine derivative of the triose. The colour intensity is directly proportional to the enzyme concentration.

Reagents:
- Fructose-1:6-diphosphate 0.05M pH 8.6
- Hydrazine 0.56M pH 8.6
- 2:4-dinitrophenolhydrazine 0.1% in 2N Hydrochloric acid
- Tris buffer 0.1M pH 8.6
- Sodium hydroxide 0.75N
- Trichloroacetic acid 15%

Procedure: Duplicate test tubes were prepared containing 1.0 ml buffer, 0.25 ml hydrazine and 1.0 ml enzyme solution. These were equilibrated at 38°C in a water bath and the reactions started by the addition of 0.25 ml fructose-1:6-diphosphate solution. For each enzyme sample a colorimeter blank was prepared and incubated without the fructose-1:6-diphosphate. After 30 minutes the reactions were stopped by the addition of 1 ml TCA. Fructose-1:6-diphosphate was added to the colorimeter blanks at this point. Tubes were centrifuged at 1200 g. for 10 minutes and 1 ml aliquots of the supernatants dispensed to test tubes containing 1 ml of sodium hydroxide. After 10 minutes at room temperature, 1 ml of 2:4-dinitrophenolhydrazine was added to each tube. The tubes were then incubated at 38°C for 10 minutes, after which 7 mls of sodium hydroxide were added to each. A characteristic brown colour developed which was read at 540 mu after 10 minutes at room temperature.

Units: A standard curve was prepared relating the optical density of the coloration induced by various dilutions of enzyme preparation to the alkali labile phosphate of the triose phosphates formed. This was done by measuring the Pi in 1 ml TCA supernatant incubated with 1 ml of 2N sodium hydroxide at room temperature for 20 minutes followed by acidification (1 ml of 2N hydrochloric acid) and measuring Pi in 1 ml TCA supernatant which had not been treated with alkali. The alkali labile Pi was taken to indicate the triose phosphates which had been formed during incubation.

The unit was the amount of enzyme which catalysed the formation of 2 u moles triose phosphate per hour, or which split 1 u mole of fructose-1:6-diphosphate per hour. The specific activity was expressed as units per mg. protein.

ATPase: Preparation of crude homogenates: Cells from exponentially growing suspension cultures were washed twice in isotonic saline buffered at pH 7.5 with Tris chloride. Thereafter the tubes containing the cells were placed in an ice cold bath and either ice cold distilled water or sucrose (0.25M) was added. In the case of distilled water, the cells ruptured almost at once. With sucrose the cell suspension required pestling before the cells ruptured. This was by a tight glass pestle revolving in a polythene centrifuge tube containing the cells, in an outer ice jacket. A speed of 1000 r.p.m. for 3 minutes was sufficient to break the cells. Quantities of protein in the region of 12 - 20 mg. per ml (36 to 60 x 10^6 cells approximately) were used for enzyme assay. Each experimental tube required 0.25 ml or 3-5 mg. protein.
Principle: ATPase activity was estimated according to the method of Kielley (1955) in which the extent of ATP hydrolysis is measured by the inorganic phosphate released.

Reagents: Adenosine triphosphate (0.02M)
Magnesium chloride (0.05M)
Tris chloride buffer (0.2M, pH 7.5)
Dilute cell homogenate
Perchloric acid (5%)

Procedure: The test tubes contained 0.1 ml of magnesium chloride, 0.25 ml of buffer, 0.30 ml of cell homogenate and 0.1 ml of water. The reaction was started by the addition of 0.25 ml of ATP to each tube. Incubation was at 28°C for 7 minutes. Under these conditions the amount of Pi released was proportional to the amount of enzyme present providing no more than about 1.25 u moles ATP were hydrolysed during the 7 minutes. Reactions were terminated by addition of 1.0 ml of perchloric acid. The following controls were included for every experiment - tubes without cell homogenate to check the spontaneous breakdown of ATP (this was in fact negligible), tubes with cell homogenates but no ATP to check the Pi content of the homogenate and any increase in this during the reaction time. Increases were never significant.

When 2:4-DNP was included, it was added at 10X its final concentration in the 0.1 ml water. The ATPase activity was calculated by subtracting the sum of the Pi found in the controls without ATP but with homogenate and that found with ATP present and homogenate absent, from the total Pi released by homogenates in the presence of ATP.

PHOSPHATE: Inorganic phosphate was measured by the method of Fiske and Subbarrow (1925). The advantage of this method is that the estimation is carried out at room temperature and therefore is suitable for the measurement of inorganic phosphate in the presence of labile phosphate esters. It measures between 0.1 and 1.0 u mole phosphate in 1.0 ml solution.

Reagents: 5.0N sulphuric acid
2.5% ammonium molybdate

Reducing Agent prepared by grinding together 0.2 g.1-amino-2-naphthol-4-sulphonic acid, 1.2g. sodium (meta) bisulphite, and 1.2 g. sodium sulphite. This was stored dry, and just before use, prepared in water.

Proccdure: Duplicate samples were estimated. 1.0 ml. of 5N sulphuric acid was added to each and mixed. This was followed by 1.0 ml of 2.5% ammonium molybdate and further mixing. Finally 0.1 ml reducing agent was quickly added with rapid mixing. The samples were left for 15 minutes at room temperature, and then each received 5.0 ml water and was read at 660 mu. Water blanks and standards containing 0.25 u mole phosphate were treated in the same way. The error was about 3%.

(3) ESTIMATION OF INTRACELLULAR ADENINE NUCLEOTIDES

This method required cells equivalent to 30-40 mg. protein - much greater than the quantities used in manometric experiments. The cells were therefore suspended in 10 ml manometric medium to give a cell to volume ratio of the order of that used in Warburg flasks, but incubated in 100 ml conical flasks. All other conditions - temperature, reciprocation rate, preincubation - were as described for Warburg experiments. All experiments were started by the addition of uncoupler and substrate together to the cell suspension.

It was found that considerable breakdown of ATP and ADP occurred during extraction unless the following adaptation of Hurlbert, Schmitz, Brumm and Potter (1954) was closely followed:- At given intervals flasks were removed from the bath and the contents rapidly poured into 10 ml centrifuge tubes which had been frozen at -20^{o}C. The cells were immediately spun down at

$0^{o}C$ and the supernatant discarded. Two mls ice cold 0.6N PCA were added to each tube, and the contents stirred with a fine glass needle. The tubes were spun at 2000 g for 5 minutes and the supernatants removed to iced centrifuge tubes. The procedure was repeated with 2 mls of 0.2N PCA twice and the supernatants combined. Maximum precipitation of the perchlorate as $KClO_4$ was obtained by neutralising to pH 6.5 with 2.5N potassium hydroxide, phenol red being used as an internal indicator. The tubes were allowed to stand at $0^{o}C$ for 5 minutes to obtain maximum precipitation before spinning. The supernatants were removed to ice cold crystallising dishes and the residues washed with 2 ml ice cold distilled water twice. The combined supernatants were frozen at $-20^{o}C$, placed in a desiccator (concentrated sulphuric acid and phosphorus pentoxide) at $4^{o}C$ and evaporated to dryness by a pump. Controls for the extraction were provided by the addition of 0.1ml quantities of 0.6mM ATP, ADP and in some cases AMP to frozen centrifuge tubes and subsequent addition of PCA and treatment as for acid-soluble cell extracts. No significant hydrolysis was found if the procedure was followed carefully. Experiments which showed hydrolysis were discarded.

After evaporation the nucleotides were dissolved in 0.15ml quantities of distilled water and immediately applied to a Whatman No. 1 chromatography paper. In most cases a total of 75 u mls of sample was applied to the paper using a 25 u ml pipette and a hair dryer blowing air at room temperature. The samples were applied about 2" apart and $1\frac{1}{2}$" from the initial solvent line. The procedure followed that of Krebs and Hems (1955), a freshly prepared solvent composed of 100 mls isobutyric acid, 55.8 mls water, 4.2 mls ammonia (0.880 s.g.) and 1.6 mls of 0.1M versene being used. The nucleotides were separated by descending solvent flow at $25^{o}C$. After the solvent front had travelled about 14" (approximately 15 hours) the paper

was removed, dried by warm air flow and examined under a 260 mu lamp. The spots were pencil ringed, cut out and eluted. Where spots could not be detected by the lamp, their positions were deduced from the markers. The elution method was that used by Dr. J. Cummins of this laboratory. Each spot (about 6 sq.cms.) was placed in the top region of a test tube with a constriction in the middle. The paper was then saturated with 0.1N hydrochloric acid and the acid spun to the bottom of the test tube on the bench centrifuge. The procedure was repeated five times and brought about the complete elution of the 260 mu absorbing material with the minimum of contamination of the eluate with paper fibres. The eluates were made up to 2.0 ml with 0.1N HCl and in most cases could be estimated spectrophotometrically without further dilution. It was confirmed that the maximum absorption occurred at 260 mu and the optical density was measured against a background eluate from the paper. This method was accurate to within 5%. Good separation was obtained, the Rf values being of the order of 0.36, 0.53 and 0.67 for ATP, ADP and AMP respectively. Unidentified spots with values of 0.15 and 0.82 were detected. Their total quantity of 260 mu absorbing material was however under 5% of the total of the three adenine nucleotides and was not routinely estimated.

Single and mixed spots of ATP, ADP and AMP (0.03 u moles in 0.1N HCl) were run with each set of samples. These ran rather slower than the extracted nucleotides but provided a useful check on nucleotide hydrolysis during extraction.

(4) GLYCOGEN

Attempts to demonstrate glycogen in L cells by the method of Carroll, Longley and Roe (1956) were inconclusive. The principle of the method is that glycogen is extracted in 5% TCA and then precipitated overnight by 95% ethanol. The glycogen is then dissolved by addition of water and estimated by the Anthrone method.

(F) RADIOISOTOPE TECHNIQUES

14-C α-Aminoisobutyric Acid

55 mM non-radioactive AIB was prepared in 84% isotonic KRP. 14-C AIB of high specific activity (gift of Dr. R. Marquis) was added to give a final count of about 0.015 uC per u mole. The radioactivity was determined by dispensing 0.1ml quantities to stainless steel planchets (1.5 cm. diameter) containing thin discs of lens tissue, drying under an infra-red lamp, and counting by a Panax D 657 counter of efficiency 17.5 - 18%. Self absorption was checked by preparing planchets with up to four times the quantity to be estimated. Approximately 5,000 counts or as near this as possible were made on all samples including cells, and the standard counting error was about 2%. Corrections were made for the background count which was low and varied between 5 and 7 per minute.

During experiments, 0.3 ml. of the 55mM AIB was added to the cells from a sidebulb fifteen minutes after addition of glucose or glucose plus uncoupler, to give a final concentration of 5.5mM. Cultures were removed from the bath at given intervals and a 2.0 ml portion rapidly removed from each to an ice cold 10 ml centrifuge tube from the deep freeze. The cells were immediately spun down at $0^{\circ}C$ and 200 g for 5 minutes. The supernatants were removed, and the cells gently washed with 8 ml. of cold KRP and spun again. After withdrawal of the supernatant and drainage of the tubes by inversion on filter paper, 2 mls of distilled water were added to each and the cells suspended. After 15 minutes duplicate samples of 0.1 ml. or 0.2 ml. were plated on planchets as described and counted. Self absorption was checked and the background corrected for. If the deviation from the mean was more than 4%, further samples were prepared and counted. Counts were related to protein, and put on a molar basis by applying the information that cells equivalent to 1 mg. protein contained 8.4 u ltrs cell

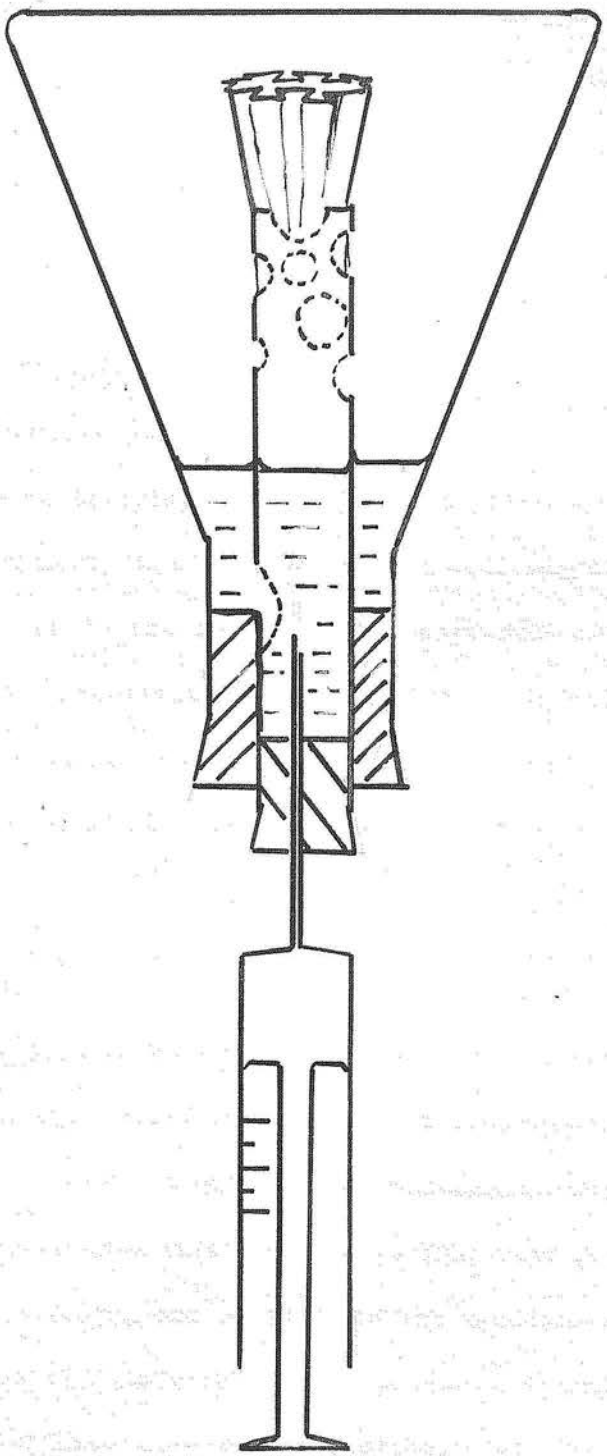
water assuming the entire cell as water. This data (Marquis) was obtained by the sucrose method for estimating intracellular space.

14-C Glucose

Uniformly labelled glucose of specific activity 21 uC per mg. was used, after dilution.

Growth Experiments: Glucose added to growth medium was used at a specific activity of 2,400 - 3,000 counts per 100 seconds per u mole. All flasks were very tightly bunged. In experiments where 14-CO_2 was collected for estimation, a device depicted overleaf was used for each flask. The filter paper in each perforated trap (perspex tubing) was saturated with 0.5 ml. .2N KOH and the trap quickly fitted into each flask. The flask was inverted carefully so that the medium ran away from the cells and collected in the neck. 0.5 ml. 2N HCl was injected through the cap into the medium to release bound carbon dioxide. The flasks were placed in the refrigerator (4^{o}C) in this position for 1.5 hours for absorption of gas. The medium was then allowed to run into a test tube through the cap, and the trap removed from the flask. The filter paper was eluted by centrifuging 5 mls. of N NaOH through it by the technique described on page 69. A stream of 5% CO_2 in air was passed through each sample for 5 minutes to provide additional carrier. Saturated barium chloride (0.5 ml) was added to each, and the precipitated barium carbonate spun down at 2000 g, washed once in 3 mls distilled water, resuspended in 0.4 ml water and dispensed quantitatively to planchets and counted with the usual precautions. For most counts with 14-C glucose an IDL scaler 1700 with automatic sample changer was used. Planchets of 1" diameter were used.

Radioactivity in the cells was measured as follows:- Cells were washed once in the cold with KRP, scraped into ice cold centrifuge tubes, and the



acid soluble fraction extracted by 0.2N PCA as described (page 60). The perchlorate was precipitated with potassium hydroxide (page 68) and the supernatant and wash concentrated by desiccation before estimating the radioactivity. The acid precipitable fraction was dissolved in 1-3 mls of 0.2N NaOH and aliquots dispensed to planchets as described.

Manometric Experiments: Typically 30 u moles of glucose (1 u mole: 2000 - 5000 counts per 100 seconds) were added with the uncoupler to the cell suspension bringing the final concentration to 10mM. On termination of the experiment, 2.0 ml portions of cell suspension were removed and the cells spun down in the cold, washed with KRP and dissolved in 0.2N NaOH to a suitable concentration for counting. If acid soluble radioactivity was to be measured, extractions with 0.2N PCA were carried out as described. If the acid-precipitable material was to be measured alone, experiments were terminated by an acid tip, the precipitated material scraped into centrifuge tubes, spun, washed and dissolved in 0.2N NaOH before counting.

Calculations on Metabolism: Normally, the measured activity in the cells and CO_2 plus that calculated for acids produced did not quite balance the 14-C glucose used. Variations in the unaccounted for fraction (which was small) suggested that this was due to CO_2 loss rather than the return of some constant proportion of 14-C to the medium as acids. It is assumed therefore that the activity of CO_2 produced is the sum of that measured plus the small unaccounted for fraction.

METABOLIC QUOTIENTS

Metabolic quotients for cells metabolising under manometric conditions have been calculated on the assumption that the amount of tissue remains unchanged during the course of the experiment. In view of the convenience and accuracy of estimation, protein content has been chosen as the standard. In fact, under the conditions used in these experiments - that is exponentially growing cells from a constant environment for manometric experiments, and exponential growth during experiments over 4 days - the content of protein, total ribose and deoxyribose per cell did not alter significantly. Five estimates on the protein content and dry weight of cells in suspension, taken at intervals over one year, show the following relationship:- 10^6 cells = 336 s.d. 14 ug. protein = 449 s.d. 28 ug. dry weight. The protein content per cell growing exponentially in monolayer cultures is not significantly different from this (page 159) but the dry weight of monolayer cells was not estimated as sufficient cells could not be grown under the defined experimental conditions, to give accurate results. On occasion, for purposes of comparison with the work of others (Discussion) metabolic quotients based on protein (mg.) have been referred to 10^6 cells by dividing by 3, and put on a dry weight basis (mg.) by dividing by 1.33. With these exceptions the notation adapted throughout is as follows:-

QO_2	ultrs. oxygen used per mg. protein hour
Q lactic acid	u moles lactic acid produced into the medium per mg. protein hour
Q α-keto acids	u moles α-keto acids " " " " " " " " " "
Q glucose	u moles glucose used per mg. protein hour
Q glucose unaccounted for	u moles glucose used unaccounted for by acid production per mg. protein hour

Metabolism has been related to ATP production as follows:-

(i) Lactic acid production: Every 2 u moles lactic acid appearing in the medium are considered as derived from 1 u mole of glucose with a net synthesis of 2 u moles of ATP.

(ii) α-Keto acid production: At pH 7.4 very little α-keto acid is produced into the medium during manometric experiments. Chromatography of the dinitrophenolhydrazones in butanol, ethanol and ammonia, according to the method of El Hawary and Thompson (1953) has shown that no less than 80% of the α-keto acids produced is pyruvic acid. The production of α-keto acids is therefore considered on the basis that no significant error arises in assuming that every 2 u moles appearing in the medium are derived from 1 u mole glucose with the utilisation of 22.4 ultrs. oxygen and a net production of 8 u moles of ATP.

(iii) Respiration rates: Calculation of ATP production from the oxygen uptake during glucose metabolism under manometric conditions is complicated by high endogenous respiration rates. As discussed on page 101 it seems likely that the endogenous substrate metabolised is glycogen. It is assumed therefore that the stoicheoimetry for glucose applies to the total oxygen uptake less that required for the appearance of α-keto acids in the medium. On this basis every 134.4 u litres of oxygen used by the cells and unaccounted for by α-keto acid production results in the net production of 38 u moles ATP.

(iv) Substrate level phosphorylations: ATP produced by substrate level phosphorylations is calculated on the basis that there is a net gain of 2 u moles for every 2 u moles of lactate or pyruvate produced into the medium, and a net gain of 4 u moles for every 1 u mole glucose respired to carbon dioxide.

The calculation of quotients for cells growing under tissue culture conditions is problematic, as it is necessary to base quotients on the average number of cells present during the experimental period. Where suspension cultures are used, this difficulty can be resolved to some extent as adequate growth curves may be obtained for each experimental flask by sampling at short

intervals. Metabolic quotients may then be calculated for every flask, and standard errors for the averages obtained. Growth curves for cells growing as monolayers can only be obtained by the replicate culture technique involving the sacrifice of several flasks for each point, which may therefore have a fairly wide scatter. With this system it is impossible to obtain a precise measurement of the growth in any single flask. Sinclair (1958) has calculated quotients on the assumption that any scatter present in the final group of tubes reflects differences in the initial inocula. Individual initial values were calculated for the tubes by multiplying the final number of cells by the ratio of the average initial number to the average final number. While this is a satisfactory approximation for the conditions of Sinclair's experiments where growth was exponential throughout and the quotients calculated over single time intervals, it has not been used in the results to be presented here for the following reasons:-

In the growth experiments to be described, it was desirable to calculate metabolic quotients over twenty-four hour periods for up to ninety-six hours by which time the growth rate was beginning to diminish. As unequal inocula often yield identical total cell harvests if cells are grown to stationary phase, it is clear that a small scatter between cell populations of cultures in the later phases of growth is not necessarily indicative that the initial scatter was correspondingly small. Conversely, in many experimental cultures containing 2:4-DNP, the scatter in the final cultures was fairly wide. It cannot however be assumed that this scatter was present in the initial populations. The action of 2:4-DNP on cell metabolism is exceedingly sensitive to hydrogen ion concentration, its action being enhanced by decreasing pH even in the physiological range. The 2:4-DNP Cell system is autocatalytic for the production of acid and the lowering of pH to a point where a small difference in inoculum size or even the presence of a cell clump in one culture could conceivably lead to grosser differences arising which would be reflected in growth.

With those difficulties in mind, no attempt has been made to work out metabolic quotients for individual cultures. In most cases, the results have been presented as follows:- The average values for the protein content, glucose utilisation, and lactic and α-keto acid production of each group of replicate cultures have been expressed in graphical or tabular form, with the standard error or standard deviation of the average stated. In Graphs where points on the same axis are almost coincident with an obvious overlap of scatter, the standard error or standard deviation has been drawn only on one side of each point, that is, above the upper point and below the lower point. Metabolic quotients have been calculated from the stated averages. The average quantity of tissue present during a period for which a quotient was to be calculated was taken as the arithmetic mean of the means of the initial and final cell cultures. The geometric mean is more accurate where cells are proliferating rapidly but in many uncoupled cultures the cells never entered an exponential growth phase and in some the cell numbers decreased. The arithmetic mean was therefore used in all cases, and it was calculated that because of the relatively short time intervals between points, any errors due to this would be no more than about 2%, well within the experimental error for the system.

STATISTICAL TREATMENT OF RESULTS

The Standard Deviation (s.d.) of a set of values has been calculated in the conventional way as the square root of the sample variance

$$s.d. = \sqrt{\frac{(x - \bar{x})^2}{n}}$$

In some cases the Standard Error of the Mean has been stated. This is the Standard Deviation divided by the square root of the number of samples.

$$s.e. = \frac{s.d.}{\sqrt{n}}$$

Frequently, tests have been made for significant differences between sample means, using the method of comparison of sample means by paired observations. (Steele and Torrie, 1960, p.78). This analysis involves the calculation of the variance of the differences between sample means, rather than among the individuals of each sample. The number of degrees of freedom is one less than the number of pairs.

$$t = \frac{d}{s.d.} = \frac{d}{\frac{S(x_1 - x_2)^2 - (S(x_1 - x_2))^2/n}{n(n - 1)}}$$

where d is the average difference between paired samples, x_1 and x_2, s.d. is the standard deviation, n is the number of pairs and n - 1 the degrees of freedom.

The probability of the differences being due to chance is determined from the position of student's t on Fisher's tables (Fisher 1948, p.174). Where P is less than 0.01, the result is considered as definitely significant.

SECTION 1

EXPERIMENTS WITH GLUCOSE AS SUBSTRATE

E X P E R I M E N T A L

Tables and Graphs are presented at the conclusion of each sub-section

GROWTH EXPERIMENTS

A. GROWTH AND METABOLISM IN 2:4-DINITROPHENOL

Experiments to assess the effects of 0.05, 0.10 and 0.15 mM 2:4-DNP on growth and metabolism were carried out as described earlier (page 43). Replicate cultures were sampled every twenty-four hours for four days and neither the medium nor the gas phase was renewed during the course of experiments. Results of the two most informative experiments are shown in Table 3 and Graphs 1 - 3. Graph 1 gives an overall picture of a single experiment showing growth (cell protein), glucose utilisation and lactic and α-keto acids production of control cultures and cultures containing 2:4-DNP. Each point represents the mean of estimations from three replicate cultures. Because of the problem of stating standard errors graphically, the information from Graph 1 is reproduced in Table 3 with these included. The information from the second experiment was essentially the same as that of Graph 1 and has not been included in this form. Graph 2, however, shows metabolic quotients for total glucose utilised, lactic acid and α-keto acid production, and "glucose unaccounted for by fermentation" in both experiments. The changes in the pH of the media, due to the metabolism set forth in Graph 1 are shown in Graph 3.

Control cultures without 2:4-DNP typically increased four-fold in the course of experiments, using about 30% of the available glucose. The rate of glucose utilisation per mg. cell protein was consistently higher at the commencement of experiments than in the later phases. This difference is significant ($p < 0.01$) if the method of comparison of sample means by paired observations is applied to data from the five control experiments shown in relation to the actions of uncouplers on growth in subsections A, B and C. The rate of production of lactic acid into the medium per mg. cell protein

showed a similar significant reduction with time, and during the fourth day the rate was typically about one half of that on the first day. Assuming that the lactic acid produced is the result of glucose metabolism, the percentage of glucose used which appeared as lactic acid fell from something over 50% on the first day to about 30% on the fourth day.

Compared with lactic acid, the production of α-keto acids into the medium was small. The greatest quantity was found at the end of the first day, and amounted to 20% or less of the lactic acid produced during this period. On subsequent days this decreased to a very low level, presumably due to oxidation by the cells.

Pyruvate forms the major portion of the α-keto acids produced into the medium (page75) and on the assumption that two molecules of lactic acid or α-keto acid represents one molecule of glucose utilised, the glucose equivalent of the acids produced into the medium has been subtracted from the glucose used, giving a figure which has been termed glucose "unaccounted for by fermentation". The significance of this quantity will be discussed later. (page84). From a consideration of the metabolic quotients of the control cultures, it is readily seen that the quotient for "glucose unaccounted for by fermentation" is less variable with time than the quotients for glucose utilised and acids produced.

The control cultures for the investigation of 2:4-DNP action therefore show a growth and metabolic pattern which is characteristic of a wide range of tissue culture cells.

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2:4-DNP produced a marked effect on growth. At the highest concentration used (0.15mM), growth was completely inhibited, and extensive necrosis occurred after the first day. In the case of 0.10 mM, a 1.6 to 1.8 fold increase was attained by the third day, after which progressive necrosis became evident. At 0.05mM, the cells grew almost exponentially

for three days, doubling their cell protein in 2.5 days, compared with 2 days for the controls. The growth rate decreased considerably on the fourth day, and over the course of the experiment the total increase in cell protein was 2.3 to 2.5 fold.

All the 2:4-DNP treated cultures showed considerably higher rates of glucose utilisation and acid production than the control cultures. During the first 24 hours, the metabolic quotients for glucose used and lactic acid produced were highest in the cultures containing 0.15mM, being about two and a half and three and a half times respectively those of the control cultures. The quotient for α-keto acids produced was about three times that for the controls, but not significantly higher than that observed for cells growing in 0.10mM. On subsequent days, the quotients for the 0.15mM treated cells decreased somewhat. It is difficult to know how much significance can be attached to this decreasing trend, however, as the quotients were calculated for what was essentially a dying population of cells, and the protein standard used made no distinction between living cells and dead cells not yet autolysed. The decreasing quotients may merely reflect a higher proportion of non-metabolising cells. This problem is discussed elsewhere (page 51). In addition, measurements of the growth and metabolism of cultures in 0.15 mM 2:4-DNP showed much larger percentage errors for the second and third day than the first. During the first twenty-four hours, the quotients for glucose used by 0.10mM and 0.05mM DNP treated cells were respectively about 2.2 and 1.6 times that of the control cultures. On subsequent days the quotients increased, this being particularly marked during the third and fourth days. As already noted, the quotients for glucose used by the control cells fell steadily during this period, and on the fourth day the quotient for glucose used by 0.10mM DNP treated cells was almost five times and the quotient for 0.05mM DNP treated cells about three and a half times that of the control cultures on

that day.

A similar pattern was noted for the rate of lactic acid production by 0.10mM and 0.05mM DNP treated cells, the respective quotients being twice and 1.6 times that of the control cells during the first day, rising to about nine times and five times the control quotient on the fourth day. A point of some interest was that by the third and fourth days, the rate of lactate production by 0.1mM DNP treated cells was greater than the rate by 0.15mM DNP treated cells on the first day.

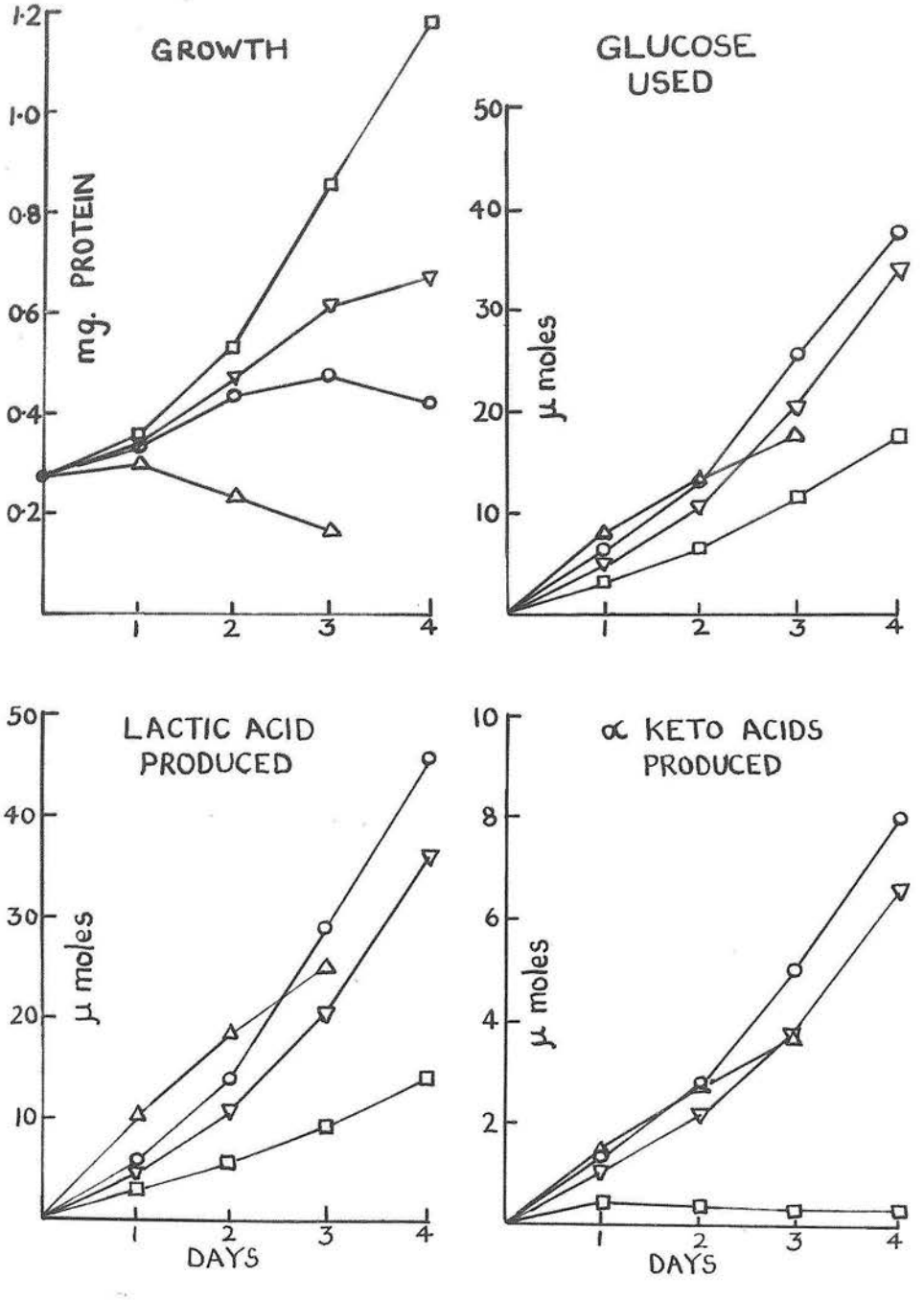
Both 0.1mM and 0.05mM DNP caused a stimulation of the rate of α-keto acid production. In the case of 0.1mM DNP treated cells, the quotient remained fairly steady at about 0.18 u moles during the first three days, rising to about 0.28 on the fourth day. A similar pattern occurred at the lower concentration of DNP, the quotient rising from about 0.12 during the first three days to 0.18 on the fourth day. The quotient for α-keto acid production by the control cells was 0.06 for the first day, but on subsequent days this fell to a negative value as the cells removed the acids from the medium.

The calculation of "glucose unaccounted for by fermentation" quotients for DNP treated cells revealed very significant differences between cells metabolising at different concentrations. Whereas 0.15mM 2:4-DNP treated cells showed higher rates for glucose used and acids produced during the first day, the quotient for "glucose unaccounted for by fermentation" was significantly lower than that of 0.10mM treated cells during this period, although about one and a half times that of the control cells. The quotients for "glucose unaccounted for" of 0.1mM and 0.05mM DNP treated cells during the first twenty-four hours were about 1.5 and 1.25 times respectively that of the control. On subsequent days the quotients for cells at the two higher DNP concentrations decreased decisively. By the third day, that for the 0.15mM treated cells was only half that of the

controls and by the fourth day the quotient for 0.1mM treated cells had decreased to almost the same as the control quotient. The earlier criticisms of metabolic quotients of cells in high DNP concentrations apply equally forcibly in this case. In contrast to the situation at the higher concentrations, the quotients for 0.05mM treated cells increased with time, and on the fourth day the quotient was equal to that for 0.1mM treated cells on the first day.

The general pattern which emerges from these experiments with regard to metabolic quotients at varying concentrations of DNP may be summed up. Whereas during the first day, stimulation of glucose utilisation and acid production is greatest by 0.15mM DNP, by the fourth day the quotients for 0.10mM treated cells are as high, if not higher, than those of the 0.15mM treated cells on the first day. Conversely, whereas on the first day the quotient for "glucose unaccounted for by fermentation" is lowest in 0.15mM treated cells, by the fourth day the quotient for 0.1mM treated cells is as low as that for 0.15mM on the first day. A comparison between the quotients for 0.05mM treated cells on the fourth day and 0.10mM treated cells on the first day shows that a similar general relationship holds.

Graph 3 shows the extent to which the pH of the media changed during the experiments. In the case of 0.15mM DNP medium, falling quotients and increasing cell deaths were accompanied by a fall in pH from 7.4 to 6.55. In the case of 0.10mM DNP the pH fell during the experiment to 6.3, and this was accompanied by rising quotients for glucose utilisation and acid production, decreasing quotient for "glucose unaccounted for" and increasing cell death. At 0.05mM DNP the pH fell to 6.45 and was accompanied by a rise in all metabolic quotients including "glucose unaccounted for".



GRAPH 1

GROWTH AND METABOLISM IN 2:4-DNP.

□ No 2:4-DNP

▽ 0.05 mM. 2:4-DNP

○ 0.10 mM. ,,

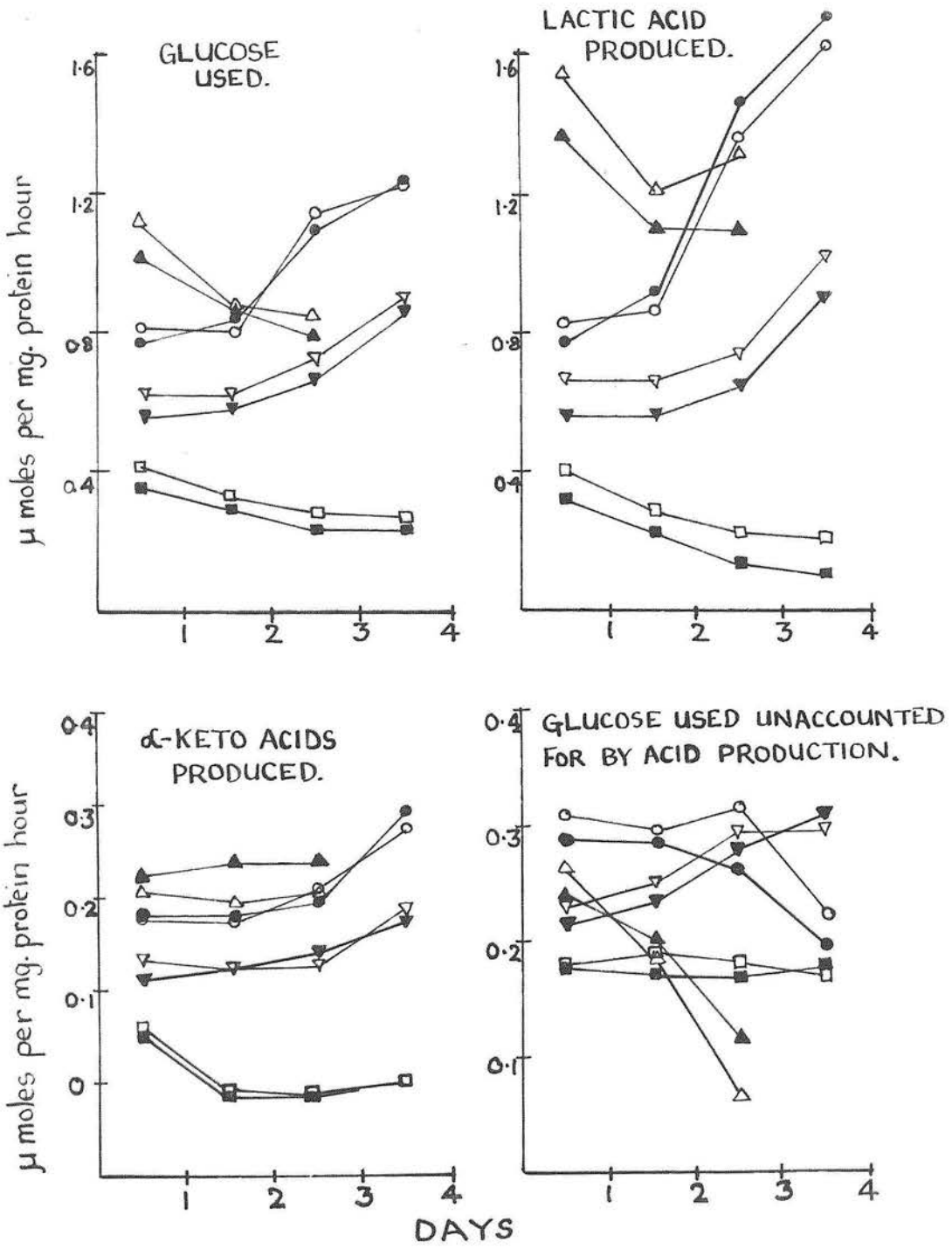
△ 0.15 mM. ,,

TABLE 3

GROWTH AND METABOLISM IN PRESENCE OF 2:4-DNP.

Time (hours)	24		48		72		96	
PROTEIN (u.g.) initials = 272 s.e.7		s.e.		s.e.		s.e.		s.e.
CONTROLS	352	7	525	20	851	17	1175	32
0.05mM	338	6	462	10	613	14	660	18
0.10mM	332	11	430	8	468	11	410	25
0.15mM	302	11	232	13	165	9		
GLUCOSE USED (u. moles)								
CONTROLS	3.10	0.07	6.49	0.18	11.20	0.19	17.62	0.52
0.05mM	4.86	0.08	10.86	0.22	20.19	0.52	33.95	1.42
0.10mM	5.85	0.13	13.26	0.47	25.37	1.18	38.45	1.20
0,15mM	7.82	0.21	13.53	0.64	17.57	1.19		
L.A. PRODUCED (u. moles)								
CONTROLS	5.05	0.07	5.91	0.12	9.45	0.12	14.25	0.36
0.05mM	4.99	0.08	11.18	0.32	20.58	0.82	36.18	1.13
0.10mM	5.97	0.28	13.91	0.49	29.0	1.09	45.59	1.13
0.15mM	10.53	0.30	18.33	0.83	24.81	0.99		
α-KETO ACIDS PROD. (u. moles)								
CONTROLS	0.46	0.03	0.38	0.01	0.30	0.01	0.30	0.01
0.05mM	1.02	0.03	2.22	0.04	3.81	0.13	6.64	0.16
0.10mM	1.32	0.03	2.80	0.12	5.05	0.13	7.96	0.22
0.15mM	1.43	0.03	2.69	0.14	3.68	0.15		

METABOLIC QUOTIENTS

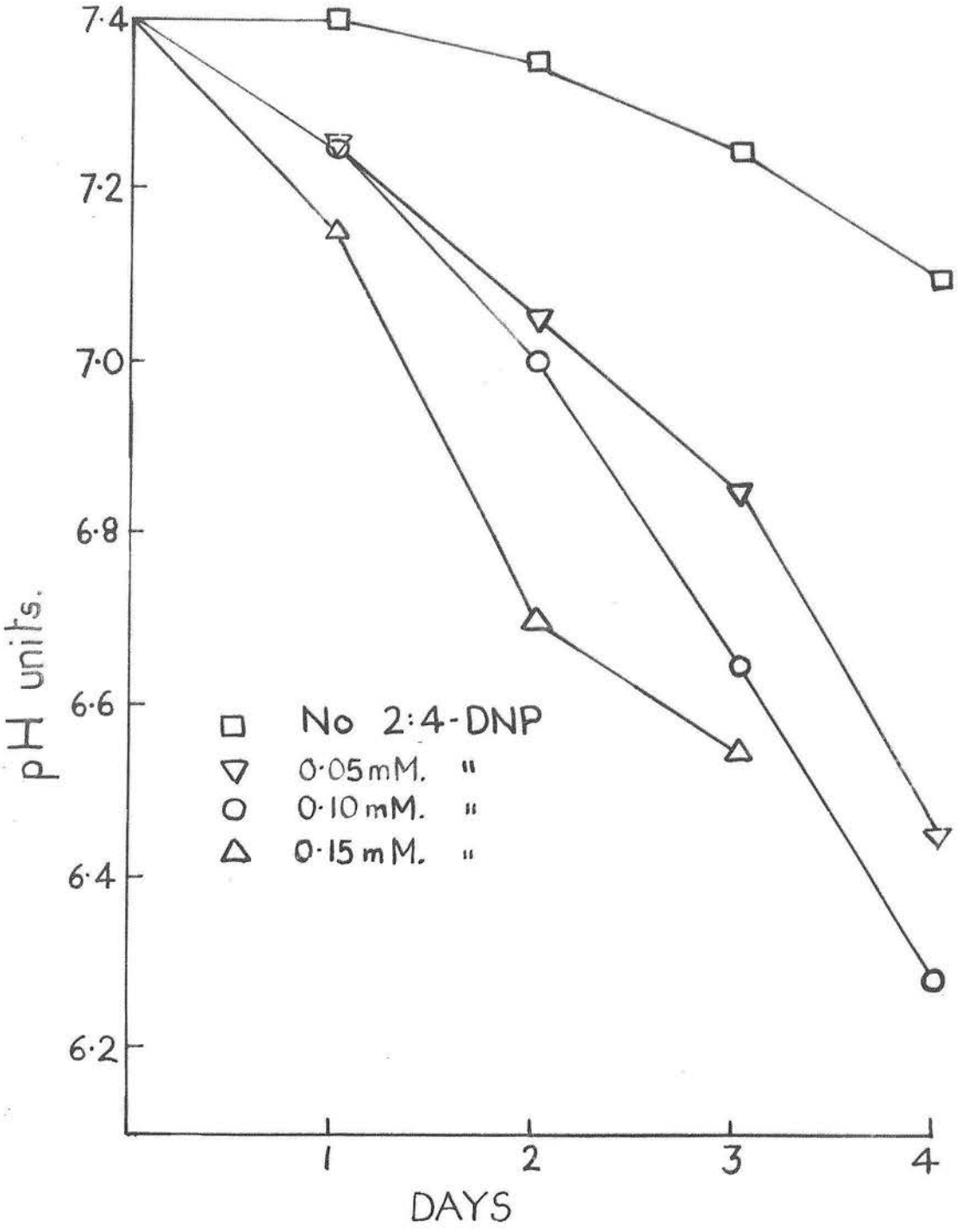


GRAPH 2

METABOLIC QUOTIENTS DURING GROWTH IN 2:4-DNP.

□ ■ No 2:4-DNP ▽ ▼ 0.05 mM. 2:4-DNP

○ ● 0.10 mM. ,, △ ▲ 0.15 mM. ,,



GRAPH 3.

CHANGES IN THE pH OF THE MEDIA DURING GROWTH IN 2:4-DNP.

B. THE UTILISATION OF 14-C GLUCOSE DURING GROWTH

The foregoing experiments have shown that the "glucose unaccounted for by fermentation" quotient is relatively constant during exponential growth. Before meaningful approximations can be made on the rate of formation of high energy phosphate bonds during growth, this quotient must be resolved into its two components - glucose respired to completion, and glucose incorporated into cellular material. Experiments using 14-C glucose were carried out to determine this. Tables 5 and 6 show the results of an experiment where replicate cultures with and without 0.10mM 2:4-DNP were sampled after 48 and 96 hours, two cultures from each being sacrificed on those occasions. As discussed on page 73, the assumption is made that the total carbon dioxide produced approximates to the sum of the activity found in the KOH-trapped portion and the activity which is unaccounted for on the completion of the 14-C balance sheet. In the experiment described, this non-recoverable fraction varied from 0 to 8.4% of the calculated value for 14-C glucose metabolised.

Cultures without 2:4-DNP showed a typical decrease with time in the percentage of glucose used which reappeared in the medium as acids. This fell from 50% over the first 48 hours to about 37% over the whole 96 hours. Conversely the percentage of glucose used which was assimilated by the cells increased slightly during the experiment, from about 11% over the first 48 hours to an average of about 14% over the last 48 hours. The actual quantities of glucose assimilated, however, correlated well with cell growth; the average number of 14-C u gm. atoms assimilated per increase of 100 ug. protein (or incorporation of approximately one u gm. atom of nitrogen into protein) was 1.82 over the first 48 hours and 1.89 over the whole experiment. The results therefore indicate that glucose carbon is assimilated at a relatively constant rate, during the exponential growth phase under these conditions. Quotients for glucose assimilated were calculated on the basis of geometric means for

cell protein over 48 and 96 hours, and found to be 0.047 - 0.043 u moles/ mg. protein hour in the first case, and 0.043 - 0.055 in the second. It is of some interest that the average rate of incorporation of 14-C glucose into acid-precipitable material (7.0 ug per mg. hour over 48 hours and 7.6 ug. per mg. hour over 96 hours) compares well with those found during manometric experiments (page 14) under very different conditions (6.6 - 10.8 ug. per mg. hour).

As with glucose assimilated, the percentage of glucose converted to carbon dioxide increased in the course of the experiment. Over the first 48 hours this was 38 - 39% and over the whole experiment 48 - 51%. The calculated carbon dioxide produced over the last 48 hours therefore accounted for about 56% of the glucose used during this period. The quotient for glucose metabolised to carbon dioxide over the first 48 hours (0.154 - 0.158 u moles per mg. hour) is sufficiently similar to that over the whole experiment (0.167-171) to conclude that exponentially growing cells oxidise glucose at a constant rate under these conditions. Since glucose was also assimilated at a relatively constant rate, these results confirm the relative constancy of the "glucose unaccounted for" quotient of previous experiments. In the 14-C experiments described, 20 - 25% of this quotient was assimilated and 75 - 80% respired. Table 7 shows the results of a14-C glucose experiment over 48 hours which essentially confirms these findings.

In general the cultures with 2:4-DNP present showed those aspects of growth and metabolism already commented upon. Whereas the total activity associated with cellular material at 48 hours was much lower than in cultures without the uncoupler, the assimilation of glucose carbon relative to increase in cell protein was not significantly different. The average number of carbon u gm. atoms assimilated per increase of 100 ug. protein was 1.93 compared with 1.83 in the controls. The "glucose unaccounted for" quotients calculated over the first 48 hours were 0.305 and 0.316 u moles per mg. hour -

figures which were in substantial agreement with those previously found (Graph 2). The glucose assimilated components of these were 0.030 and 0.032, that is, about 10 per cent. Comparison with the results for the control cultures indicates therefore that 0.10 mM 2:4-DNP inhibited the rate of 14-C assimilation over 48 hours by approximately 33%. Since the rates for glucose respired to carbon dioxide were 0.275 and 0.284 u moles per mg. hour, respiration rate was stimulated by about 70% by the uncoupler.

Interpretation of the results from the cultures sacrificed at 96 hours is complicated by the fact that the cell populations are lower than they were at 48 hours. Inevitably therefore, 14-C activity associated with cellular material has been lost to the medium and forms part of the "unaccounted for" fractions. This figure cannot be computed with any confidence and the assumption that the total carbon dioxide produced is the sum of the activity in the KOH fraction and the "unaccounted for" fraction is untenable in this case. Despite the difficulties of stating either the total cell activity or the total carbon dioxide produced precisely, it is clear from Table 6 that, during the third and fourth days the oxidation of glucose to carbon dioxide by the cultures can be no more than 50% of that during the first two days. This confirms the falling "glucose unaccounted for by fermentation" quotient of previous experiments (page 82).

TABLE 5

UTILISATION OF 14-C GLUCOSE DURING GROWTH

	48 hours				96 hours			
	Culture 1		Culture 2		Culture 1		Culture 2	
Cell Protein (ug.)	542		560		1040		1107	
Glucose used (u moles)	7.48		7.32		17.56		18.12	
Lactic Acid produced	6.80		7.02		12.68		12.95	
α-keto Acids produced	0.44		0.40		0.22		0.25	
14-C Activity	cpm	%	cpm	%	cpm	%	cpm	%
Glucose used	13830	100	13534	100	32468	100	33504	100
Total acids produced	6693	48.4	6860	50.7	11925	36.7	12203	36.4
Acid Sol. Fraction	204		209		422		503	
Acid PPT Fraction	1424		1268		3151		4578	
Total Cell Count	1628	11.8	1477	10.9	4173	12.8	5081	15.1
Carbon Dioxide	4608	33.3	5053	37.3	13677	42.1	16232	48.4
Unaccounted for	901	6.5	144	1.1	2693	8.4	- 12	0
C assimilated per increase of 100 mg. Protein	1.82 ugm atoms over 48 hours				1.89 ugm. atoms over 96 hours			

Each flask received 15.05 mM glucose (total activity 11,3100 c.p.m.)

Initial cell protein - 274 ug. s.d. 8.

TABLE 6

UTILISATION OF 14-C GLUCOSE DURING GROWTH IN 0.10mM 2:4-D N P.

	48 hours				96 hours			
	Culture 1		Culture 2		Culture 1		Culture 2	
Cell Protein (ug.)	410		432		396		361	
Glucose used	14.04		14.30		33.90		32.65	
Lactic acid produced	15.13		15.02		46.32		43.04	
α-keto acids produced	3.02		3.24		7.44		7.85	
14-C Activity	cpm	%	cpm	%	cpm	%	cpm	%
Glucose used	25960	100	26441	100	62681	100	60370	100
Total Acids	16770	64.6	16882	63.8	49706	79.2	47039	77.9
Acid Sol. Fraction	163		142		138		165	
Acid PPT Fraction	773		867		1008		873	
Total Cell Count	936	3.6	1009	3.8	1146	1.8	1038	1.7
Carbon Dioxide	7801	30.0	7320	27.7	10922	17.4	9818	16.2
Unaccounted for	453	1.8	1230	4.7	907	1.6	2475	4.1
C assimilated for increase of 100 ug. Protein	1.93 ugm. atoms over 48 hours							

TABLE 7

14-C GLUCOSE METABOLISM DURING GROWTH

Initial cell protein	312 ug. s.d.12			
Final cell protein (48 hours)	629		643	
Glucose used	7.33		7.30	
Lactic acid produced	7.18		7.25	
α-keto acid produced	0.32		0.24	
14-C activity	c.p. 100 secs.	%	c.p. 100 secs.	%
Glucose used	17614	100	17542	100
Total acids produced	9007	51	8996	52
Acid-soluble fraction	292		256	
Acid-precipitable fraction	1963		1759	
Total cell count	2255	13	2015	11
Carbon Dioxide	5642	32	5609	32
Unaccounted for	710	4	922	5
C assimilated per increase of 100 ug. protein	1.64 ugm. atoms			

Specific activity : 1 u mole glucose = 2403 c.p. 100 secs.

C. THE INFLUENCE OF pH ON GROWTH AND METABOLISM IN 2:4-DINITROPHENOL

The foregoing experiments have shown that there is limited cell growth for 2 - 3 days in 0.10 mM 2:4-DNP, followed by necrosis. The onset of necrosis is accompanied by a greatly increased glycolytic rate, a decrease in respiration rate, and a marked fall in pH. Experiments were carried out to assess the effects of 0.10 mM uncoupler on growth and metabolism when the pH of the medium was maintained between 7.2 and 7.4 by titration (page 46). Graph 4 shows the effects of stabilisation of pH on 2:4-DNP action on growth over 4 days. The data presented is from 2 separate experiments, each point being the mean of the protein content of 4 replicate cultures. Standard deviations are shown. In the titrated cultures, the initial rate of growth was maintained over the whole experimental period, increase in cell protein being exponential throughout with a doubling time of about 70 hours. The toxic effects of 2:4-DNP were therefore offset considerably by maintenance of the pH at physiological level. Graph 5 shows the extent to which pH was controlled by the titration technique. A further result of maintaining pH above 7.2 was that the DNP induced metabolic quotients remained fairly constant with time, and did not show the characteristic changes of the untitrated 2:4-DNP containing cultures. Data from the two experiments is presented in Tables 8 and 9, each quotient being calculated from measurements of the glucose, lactic acid and α-keto acids present in pooled medium from the four cultures sacrificed at each point. As the metabolic quotients from both experiments are sufficiently similar to average, the means are expressed graphically, with the actual deviations included (Graph 6).

Although the above experiments show considerable differences in growth and metabolism where cells are exposed to the uncoupler at constant and falling pH values, the controls without 2:4-DNP are inadequate to provide further analysis of the situation as they do not indicate growth and metabolism in

cultures without the uncoupler at pH values similar to those normally induced by its presence. Previously obtained data had indicated that very slow growth would continue at pH values as low as 6.4 for a limited time, but these results were thought inadequate as they gave no indication of the cumulative effects of a steady lowering of pH with time. Experiments therefore were carried out to compare growth and metabolism of 0.10 mM 2:4-DNP treated cells with that of cells without the uncoupler passing through comparable pH changes. This was achieved by acid titration approximately every twelve hours (page 47). Graph 7 illustrates the results of an experiment in which this technique was employed, and shows the close approximation obtained between the pH values of titrated cultures without the uncoupler and those of untitrated cultures containing 0.10 mM 2:4-DNP.

Measurements of growth and metabolism in this experiment are presented in Graphs 8 and 9. As in the previous experiment, metabolic quotients were calculated from analysis of medium pooled from 4 replicate cultures. Over the first 48 hours, during which the pH values of the untitrated and titrated cultures without uncoupler were reduced to 7.25 and 7.05 respectively, there was no significant difference in the growth rate. During the third day, the pH of the titrated cultures was further reduced to 6.7, whereas that of the untitrated cultures remained about 7.2. This induced fall in pH reduced the growth rate over this period by about 40 per cent. During the fourth day, the pH of the titrated cultures was reduced to 6.4, compared with a final pH of 7.1 in the control cultures, and the growth rate was in the region of 35% of that of the control cultures which were in fact no longer growing logarithmically. The mean increase in the protein of the titrated cultures during the fourth day was therefore small (761 ug. s.e. 19 to 838 ug. s.e. 8). On repeating the experiment the mean increase on the fourth day was 715 ug. s.e. 10 to 795 ug. s.e. 17). No further experiments were done, and on the

above results growth on the fourth day must be regarded as being scarcely significant. In contrast, the cultures in 0.10 mM 2:4-DNP showed the usual small decrease on the fourth day, highly significant when considered with the several other experiments reported previously.

The most significant feature of the metabolism of the acid titrated cultures compared to that of untitrated controls without 2:4-DNP was a reduction in the rate of lactic acid production during the second and third days, and a significant utilisation of the lactic acid in the medium during the fourth day. Throughout the experiments, the levels of α-keto acids in the media were consistently lower in the case of the acid titrated cultures. As this was noted from the first day, it suggests that the production of α-keto acids is rather more sensitive to changes in pH than lactic acid production.

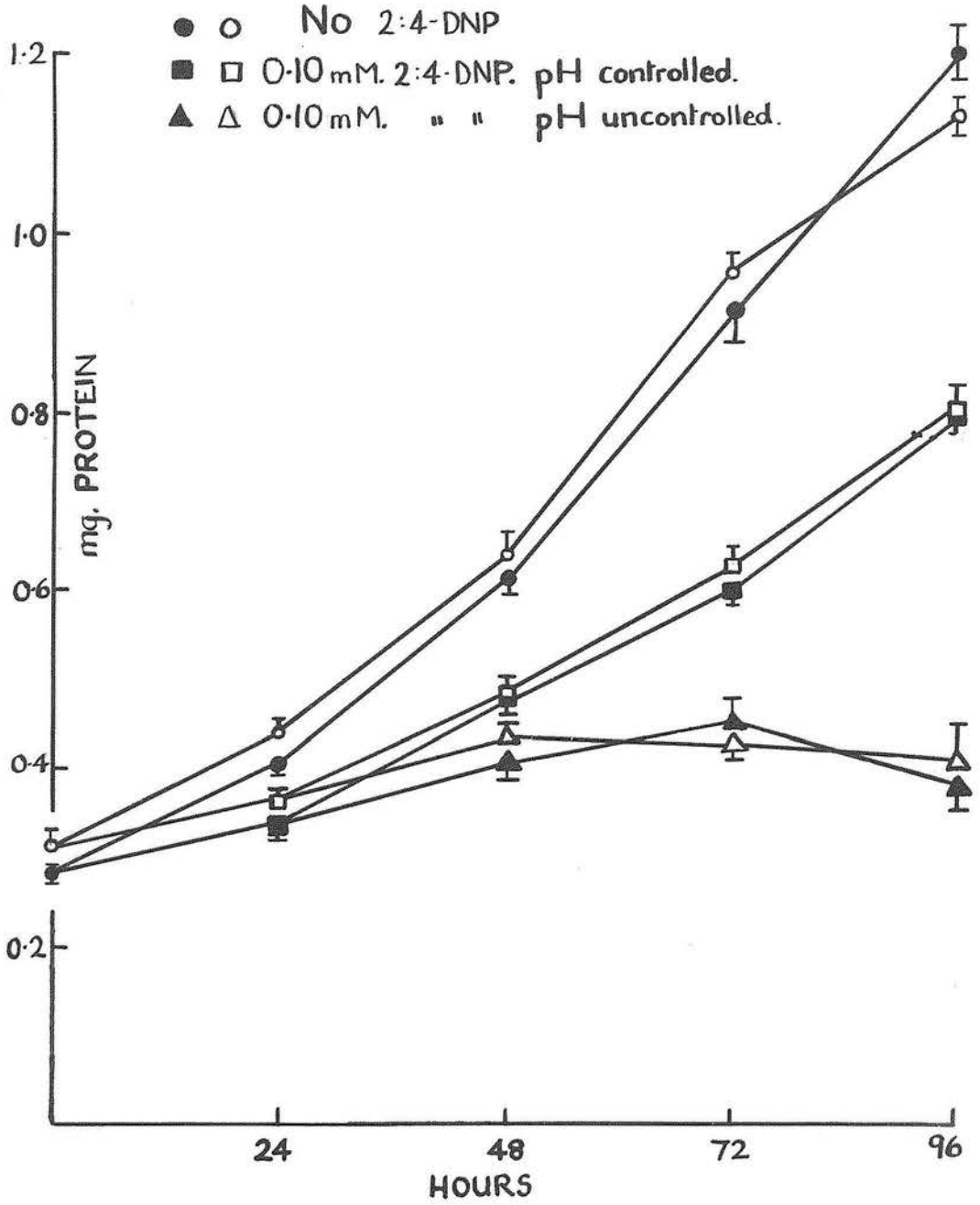
The results of acid titration experiments therefore indicate that the observed inhibition of growth and marked necrosis on the third and fourth days of growth in 0.10 mM 2:4-DNP could be due in large measure directly to the lowering of the pH of the medium by the products of cell metabolism. In this respect it is of note that lowering of pH in the absence of 2:4-DNP on the fourth day, reduced the quotient for "glucose unaccounted for" somewhat in these experiments. Lowering of pH in the absence of the uncoupler influenced all other metabolic quotients in precisely the opposite way to lowering of pH when 0.10mM 2:4-DNP was present.

The results therefore suggest that necrosis on the fourth day of growth in 2:4-DNP is partly the result of a direct effect of unphysiological pH on the cells and partly due to the accentuation of 2:4-DNP action by falling pH.

With the finding that stabilisation of pH enabled cells to grow in 0.10mM 2:4-DNP, it became important to establish the greatest concentration of uncoupler which permitted cell survival at pH 7.4 and obtain data on metabolism in this case.

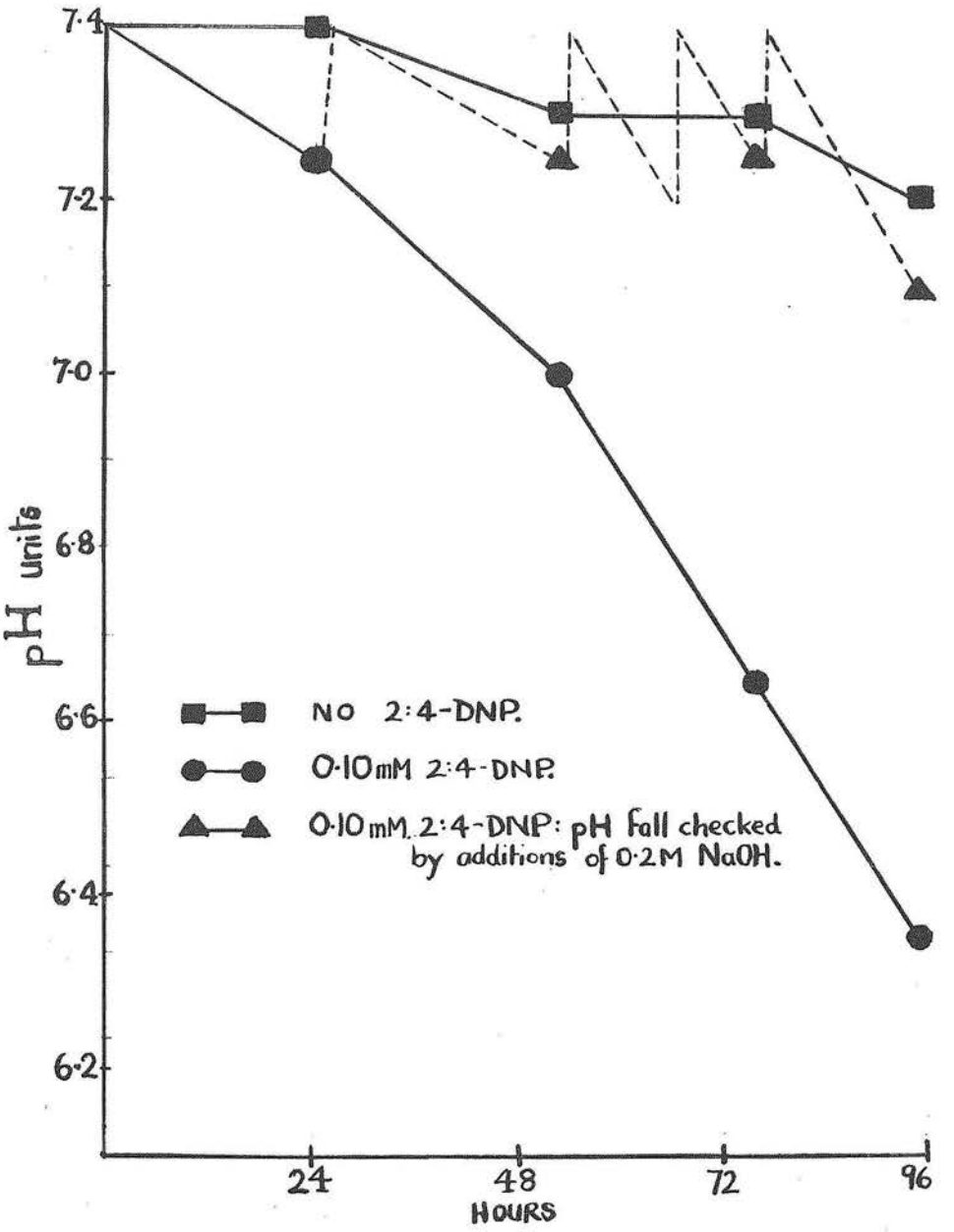
It was found that maintenance of pH did not prevent widespread necrosis within 2 - 3 days where 0.15mM uncoupler was used. Cells survived however to a varying extent in 0.125mM if the pH was maintained about 7.4 (Table 4). Furthermore, the quotients for acids produced and glucose unaccounted for by acid production remained relatively constant over 4 days. The results presented in Table 4 were derived from 3 replicate cultures (controls) and 4 replicate cultures (experimentals) sacrificed each day.

It was in fact difficult to predict the precise behaviour of cells at this concentration of uncoupler. In some cases limited growth occurred (page93). In most cases, however, cells survived with little change for up to 14 days if the pH was maintained about 7.4. Decrease in pH induced rapid necrosis.



GRAPH 4.

EFFECTS OF MAINTAINING pH ABOVE 7.2 DURING GROWTH IN 2:4-DNP.



GRAPH 5.

CONTROL OF pH DURING GROWTH IN 2:4-DNP.

TABLE 8

EFFECTS OF pH ON THE METABOLIC QUOTIENTS OF CELLS GROWING IN 0.1mM 2:4-DNP.

EXPERIMENT A

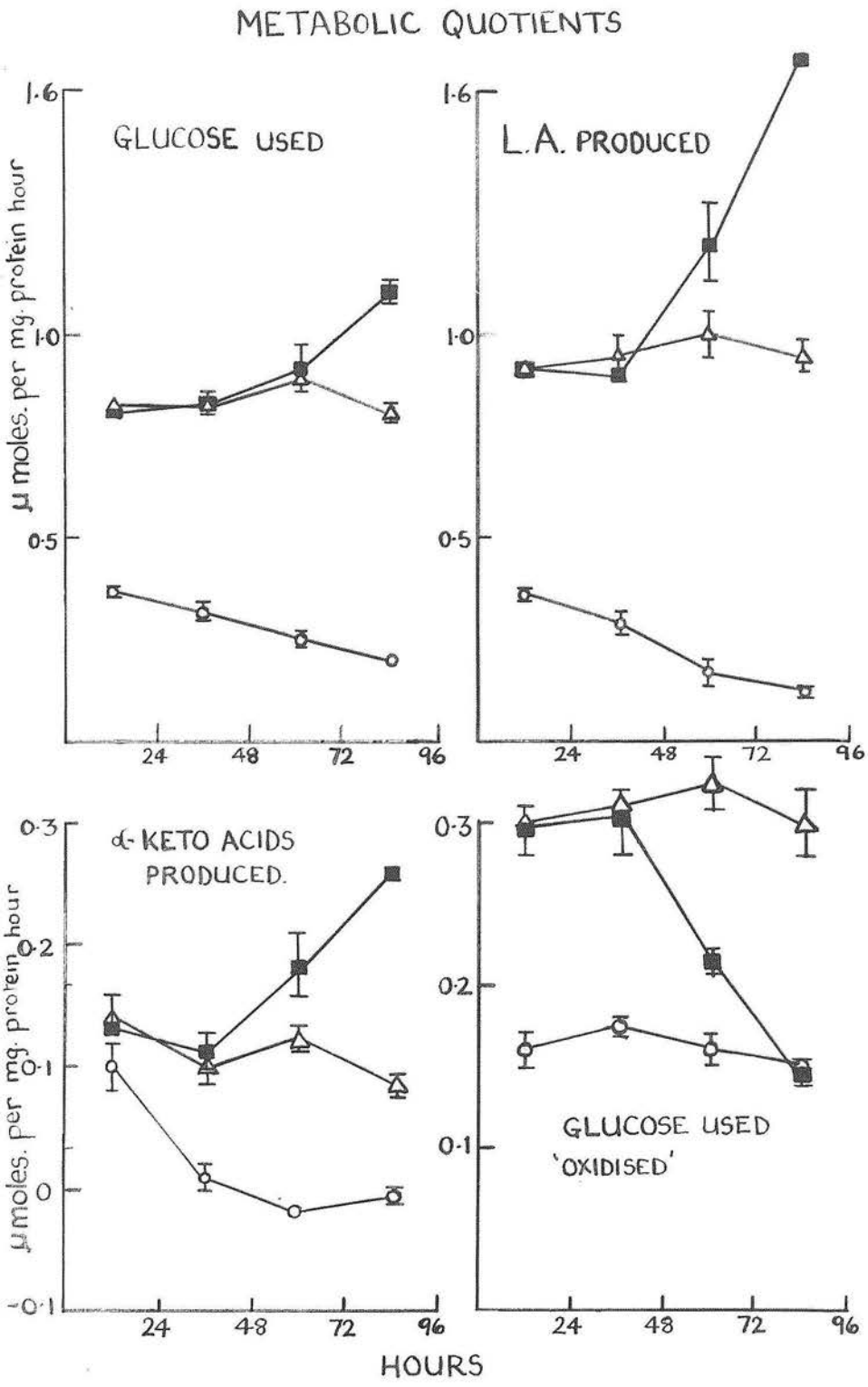
	0 - 24	24 - 48	48 - 72	72 - 96
Q Glucose used (u moles/mg.hr.)				
Control Cultures No 2:4-DNP	0.38	0.31	0.24	0.21
2:4-DNP pH uncontrolled	0.82	0.81	0.86	1.10
2:4-DNP pH controlled	0.83	0.86	0.94	0.82
Q Lactic acid produced (u moles/mg.hr.)				
No 2:4-DNP	0.37	0.26	0.14	0.13
2:4-DNP pH uncontrolled	0.91	0.90	1.13	1.65
2:4-DNP controlled	0.89	1.00	1.06	0.99
Q α-keto acids produced (u moles/mg.hr.)				
No 2:4-DNP	0.12	0.02	-0.02	-0.01
2:4-DNP pH uncontrolled	0.16	0.13	0.21	0.26
2:4-DNP pH controlled	0.16	0.08	0.13	0.07
Q "Glucose unaccounted for" (u moles/mg.hr.)				
No 2:4-DNP	0.15	0.17	0.17	0.15
2:4-DNP pH uncontrolled	0.31	0.28	0.21	0.14
2:4-DNP pH controlled	0.30	0.32	0.34	0.32

TABLE 9

EFFECTS OF pH ON THE METABOLIC QUOTIENTS OF CELLS GROWING IN 0.1mM 2:4-DNP

EXPERIMENT B

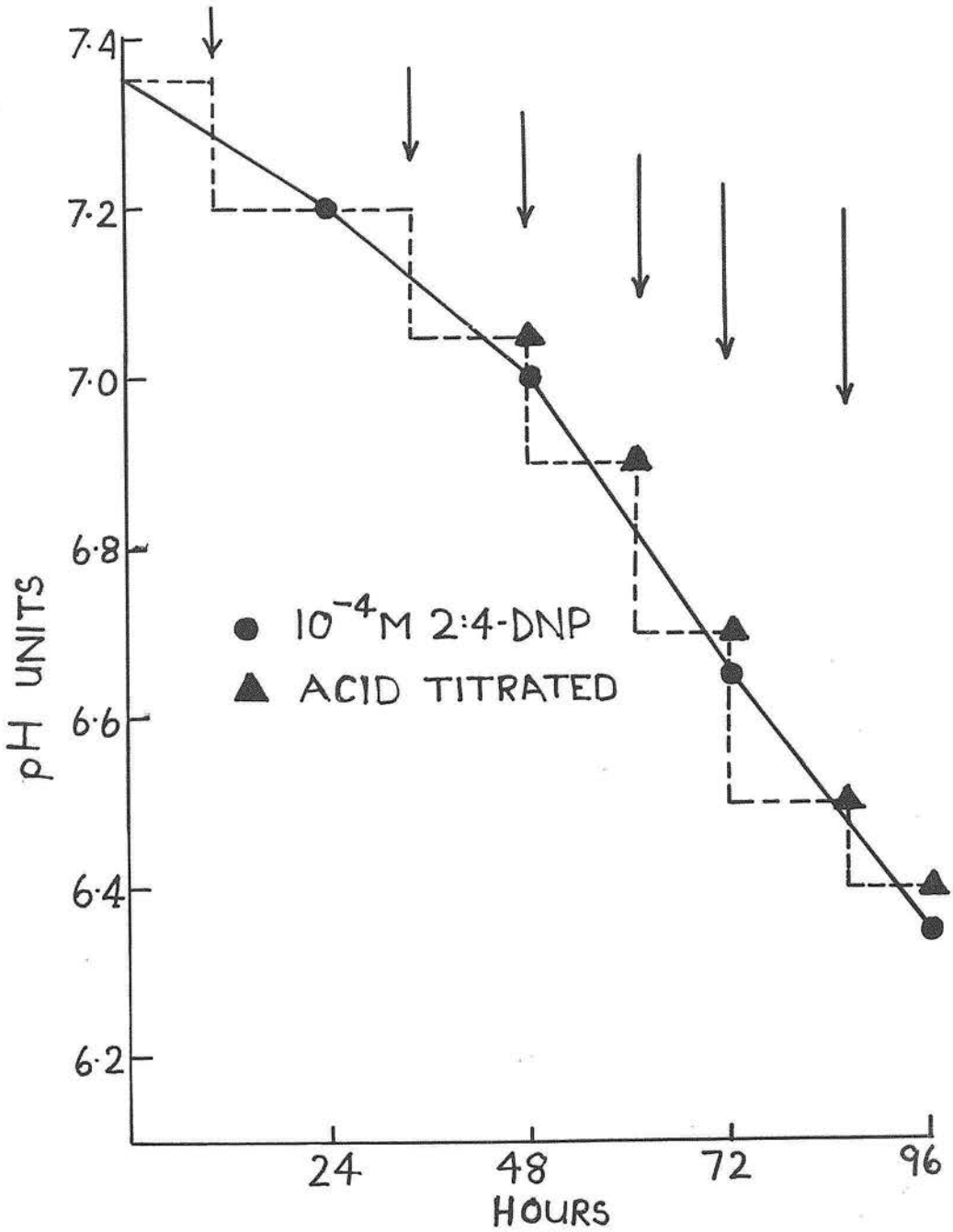
	0 - 24	24 - 28	48 - 72	72 - 96
Q Glucose used (u moles/mg.hr.)				
Control Cultures No 2:4-DNP	0.36	0.34	0.27	0.21
2:4-DNP pH uncontrolled	0.81	0.85	0.98	1.13
2:4-DNP pH controlled	0.84	0.82	0.86	0.78
Q Lactic acid produced (u moles/mg. hr.)				
Control cultures No 2:4-DNP	0.36	0.32	0.21	0.12
2:4-DNP pH uncontrolled	0.93	0.91	1.33	1.69
2:4-DNP pH controlled	0.95	0.90	0.96	0.91
Q α-keto acid produced (u moles/mg. hr.)				
Control cultures No 2:4-DNP	0.08	0.00	-0.02	-0.01
2:4-DNP pH uncontrolled	0.10	0.10	0.16	0.26
2:4-DNP pH controlled	0.12	0.11	0.12	0.09
Q "Glucose unaccounted for" (u moles/mg. hr.)				
Control cultures No 2:4-DNP	0.17	0.18	0.16	0.15
2:4-DNP pH uncontrolled	0.28	0.33	0.22	0.15
2:4-DNP pH controlled	0.29	0.31	0.31	0.28



GRAPH 6.

EFFECTS OF MAINTAINING pH ABOVE 7.2 ON METABOLIC QUOTIENTS DURING GROWTH IN 2:4-DNP.

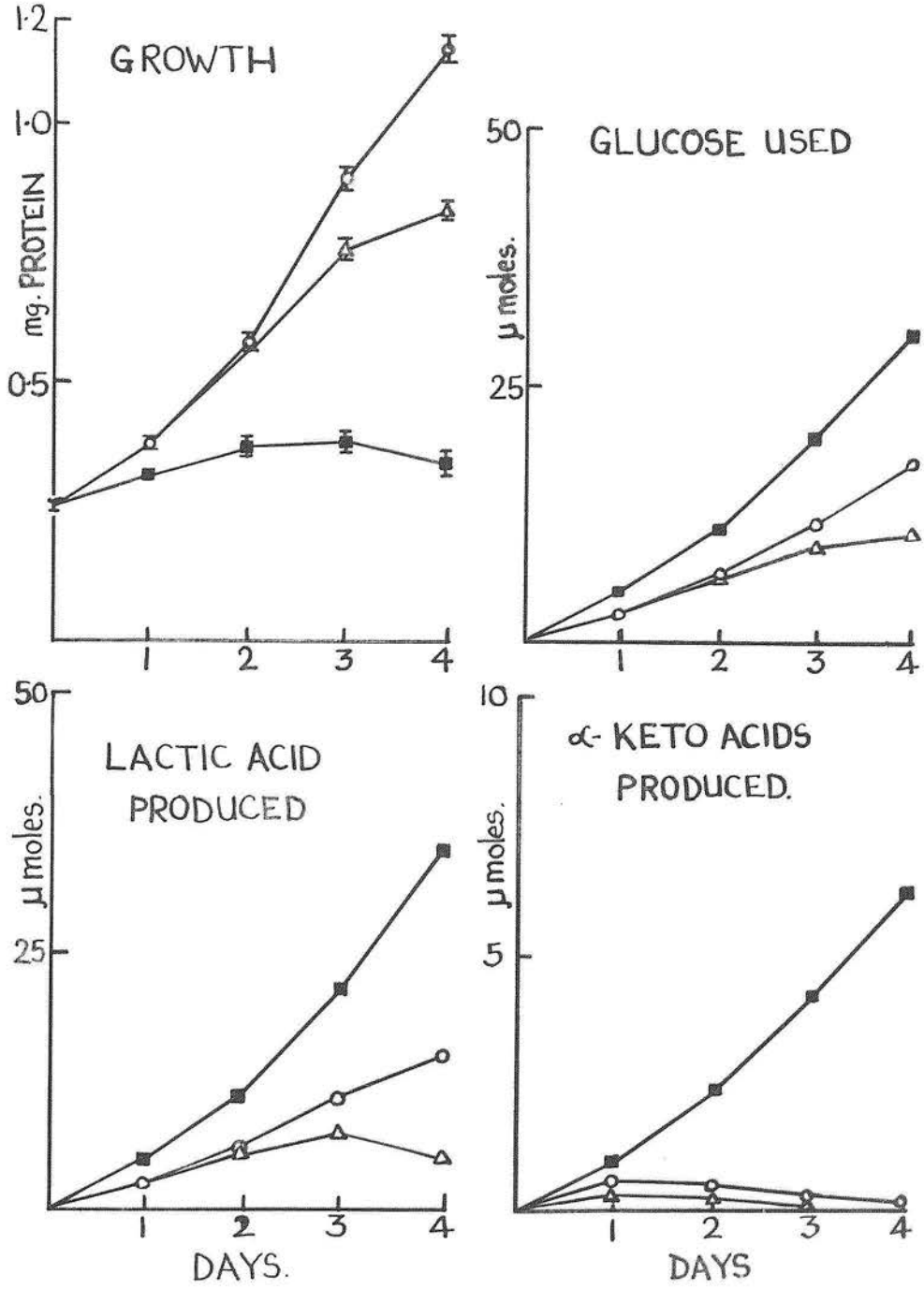
○ No 2:4-DNP; ■ 0.1 mM. pH uncontrolled; △ 0.1 mM. pH controlled:



GRAPH 7

ACID TITRATION OF CULTURES WITHOUT 2:4-DNP, TO SIMULATE THE DECREASING pH NOTED DURING GROWTH IN 2:4-DNP.

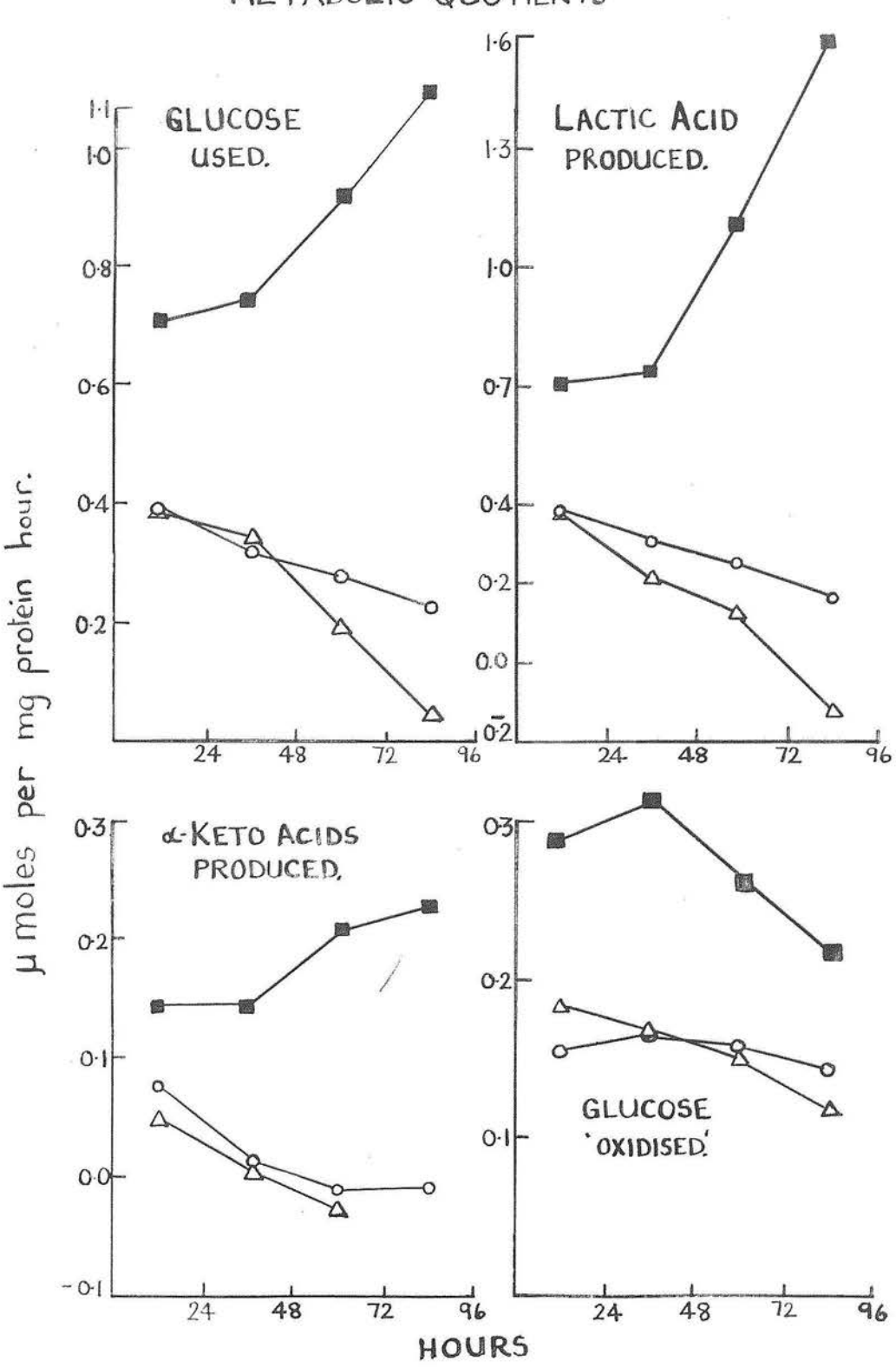
The arrows indicate acid addition.



GRAPH 8.

THE EFFECTS OF SIMULATING THE DECREASING pH NOTED DURING GROWTH IN 2:4-DNP ON THE GROWTH AND METABOLISM OF CELLS GROWING WITHOUT 2:4-DNP.

○ No 2:4-DNP; △ No 2:4-DNP, acid titrated; ■ 0.1 mM. pH uncontrolled.



GRAPH 9.

THE EFFECTS OF SIMULATING THE DECREASING pH NOTED DURING GROWTH IN 2:4-DNP, ON THE METABOLIC QUOTIENTS OF CELLS GROWING WITHOUT 2:4-DNP.

○ No 2:4-DNP; △ No 2:4-DNP, acid titrated; ■ 0.1mM. pH uncontrolled.

TABLE 4

THE EFFECTS OF 2:4-DNP (0.125mM) WHERE pH WAS MAINTAINED AT 7.2 - 7.4 OVER 4 DAYS

	1	s.d.	2	s.d.	3	s.d.	4 days	s.d.
PROTEIN (Initial: 282 ug.) Control (ug.) s.d. 10	410	17	596	20	882	31	1124	15
2:4-DNP (ug.)	310	16	302	22	285	7	315	18
	0-1		1-2		2-3		3-4 days.	
GLUCOSE USED u moles per m.g. protein hour Control	0.40		0.30		0.28		0.27	
2:4-DNP	0.98		1.04		1.08		0.97	
LACTIC ACID PRODUCED u moles per m.g. protein hour : Control	0.36		0.29		0.24		0.18	
2:4-DNP	1.24		1.37		1.48		1.29	
α-KETO ACIDS PRODUCED u moles per m.g. protein hour: Control	0.06		-0.02		0.0		0.0	
2:4-DNP	0.16		0.19		0.14		0.18	
GLUCOSE "UNACCOUNTED FOR" u moles per m.g. protein hour: Control	0.18		0.16		0.16		0.17	
2:4-DNP	0.29		0.25		0.27		0.24	

D. LONG TERM GROWTH IN 2:4-DINITROPHENOL

With the finding that cell necrosis induced by 0.1mM 2:4-DNP could be prevented by maintaining the pH of the medium at about 7.4, experiments were conducted to determine whether cells could grow in 2:4-DNP for prolonged periods under these conditions. A very approximate but convenient way of estimating growth rate over long periods of time is to record the frequency of subculturing, where this is done when a specific cell density is achieved. This method was used as follows:- All cultures were subcultured as soon as a complete monolayer was formed, and each daughter culture received 25% of the cells. With this procedure, inoculated cells increased approximately four-fold before subculture and never entered stationary phase. To prevent any constituents of the medium from becoming limiting, the pH was controlled by complete changes of medium rather than by the addition of alkali. To control as far as possible any lag phases in growth due to introduction of fresh medium, the medium was changed in every culture, including those without 2:4-DNP, at the same intervals. Typically the media was changed on the second and fourth days after subculturing and every subsequent day until the next subculture. The results of an experiment involving growth in 0.05, 0.10 and 0.125 mM are shown on the following page.

Subculture Number	Days between subcultures			
	No 2:4-DNP	0.05mM	0.10mM	0.125mM
1	4 4	5	7 7	8 10
2	4 4	6	7 7	10 8
3	3 3	6	6 8	10 10
4	4 4	6	7 6	8 died
5	4 4	5	7 7	died
6	4 3	5	6 7	
7	3 3	6	7 died	
8	4 4	6	7	
9	3 3	4	6	
10	4 4	7	7	
11	4 4	6		
12	3 4	6		
13	4 3			
14	4 4			
15	3 3			
16	4 4			
17	3 3			
18	4 4			
MEAN	3.7 3.7	5.6	6.6 6.6	9 9

This shows decisively that prolonged growth can be sustained in medium containing up to 0.10mM 2:4-DNP. At this concentration the MGT was approximately 3.3 days compared with 1.85 days for the controls, and a 100,000-fold increase of the initial inoculum took place over the ten weeks the cells grew. Subcultures from one of the initial 0.10mM 2:4-DNP treated cultures died after 5 weeks. This was due to an error in the changing schedule and the subsequent exposure of the cells to low pH for two days. It was consistently observed that with increasing time in 2:4-DNP, cells became increasingly difficult to recover from even relatively short exposure to low pH. In addition, when cell numbers were reduced for any reason to below about 0.12×10^6 cells per ml., even at pH above 7.2, growth seldom took place in the presence of 0.10mM uncoupler. Control cultures invariably grew under such conditions.

At 0.125 mM 2:4-DNP, cell growth was slow and unpredictable. In one instance cultures grew for 40 days with a MGT of about 4.5 days, indicating an approximate 250-fold increase of the initial inoculum before degeneration. In the other line, growth ceased after about 3 weeks, and this was more typical of other attempts to grow cells at this concentration.

On three occasions during ten weeks growth in 0.1mM 2:4-DNP, namely after 15, 32 and 53 days, analyses of the proportions of total ribose, deoxyadenosine equivalents, and protein per cell were undertaken to determine whether any gross changes had occurred. The results are shown in Table 10. They indicate that insofar as the parameters measured are important, the growth in 0.10 mM 2:4-DNP was essentially balanced growth. Where the pH was maintained near 7.4, the growth rate as determined by frequency of subculturing remained relatively constant over ten weeks in 2:4-DNP with no indication of adaptation to the uncoupler. A more precise assessment of the growth potential of the cells was, however, obtained by controlled growth experiments over four days. After 35 days and 60 days of growth in medium

containing 0.10mM, and after 40 days in medium with 0.05mM, 2:4-DNP cells were washed quickly in medium without uncoupler, and their growth with and without 0.10mM 2:4-DNP compared with the growth of cells which had not been exposed to the uncoupler. The results showed that there was no difference in the behaviour of cells which had been previously grown in the uncoupler and those which had not (Tables 11 and 12). These findings demonstrate that there is no significant adaptation or selection to 2:4-DNP which is manifest in either growth or glycolytic rates. Furthermore, they underline the suggestion already made that growth in the uncoupler is balanced, and can readily be released from the suppression which uncoupling imposes. The absence of a significant lag phase on inoculating 2:4-DNP treated cells into medium without the uncoupler indicates that 2:4-DNP is not firmly bound to sites of action within the cell to the extent that it is not in ready equilibrium with the external medium.

Growth in 0.05mM uncoupler was accompanied by significant increases in lactic dehydrogenase and aldolase activity. Graph 10 shows that this was apparent from the third day after addition of 2:4-DNP and reached a maximum on the fourth day. Each point of the graphs represents the mean of 3 determinations on a single culture. The average increase in LDH activity induced by 0.05mM from the third to the sixteenth day was 30% s.d. 10%. The average increase for aldolase activity from the fourth to the eighteenth day was rather higher than that for LDH at 54% s.d. 9%. Although small increases in the activity of these enzymes were observed in cells growing in 0.10 2:4-DNP, these were not clearly significant. No significant increase in Malic dehydrogenase activity was induced by either concentration of 2:4-DNP.

TABLE 10

Day of Experiment	Cultures	No.	PROTEIN mg./5x10^6 cells	RIBOSE ug./5x10^6 cells	DESOXYADENOSINE ug./5x10^6 cells
15th	Control	3	1.720 s.d. 0.080	80 s.d. 7	45 s.d. 3
	0.05mM	2	1.650 0.055	65 5	43 2
	0.10mM	3	1.832 0.092	72 4	38 3
32nd	Control	3	1.682 s.d. 0.100	86 s.d. 3	52 s.d. 2
	0.10mM	3	1.712 0.020	77 0	46 2
53rd	Control	2	1.612 s.d. 0.064	72 s.d. 4	43 s.d. 0
	0.05mM	2	1.665 0.038	72 4	45 2
	0.10mM	3	1.634 0.072	68 3	47 2

TABLE 11

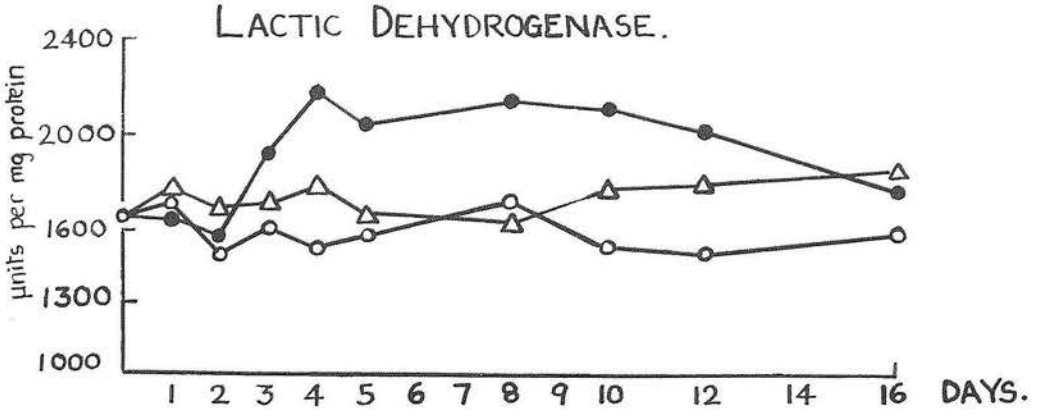
GROWTH AND GLYCOLYSIS AFTER 40 DAYS EXPOSURE TO 0.05mM DNP

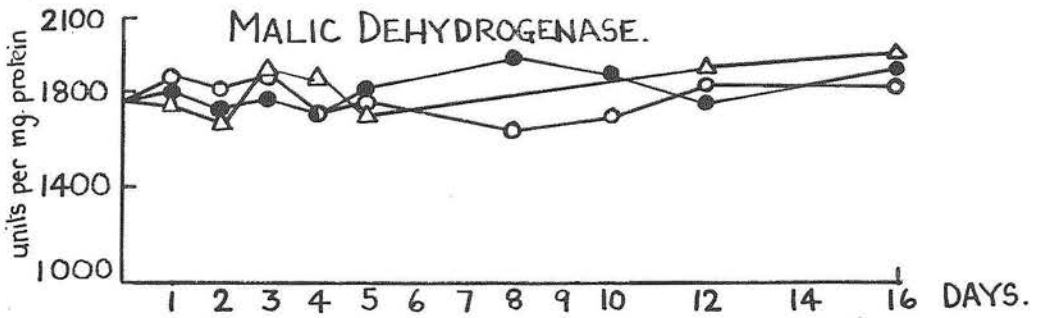
2:4-DNP	-		-		0.1mM		0.1mM	
Prior Exposure to 0.05mM	-		40 days		-		40 days	
Days	Protein	QLA	Protein	QLA	Protein	QLA	Protein	QLA
0	252 ± 10		272 ± 10		252 ± 10		272 ± 10	
1	400 ± 14	0.36	431 ± 9	0.40	353 ± 10	0.82	375 ± 12	0.87
2	541 ± 8	0.30	579 ± 15	0.30	429 ± 21	0.99	418 ± 16	1.04
3	829 ± 16	0.24	820 ± 14	0.21	463 ± 11	1.23	472 ± 7	1.40
4	1124 ± 28	0.17	1235 ± 29	0.18	397 ± 13	1.73	371 ± 17	1.69

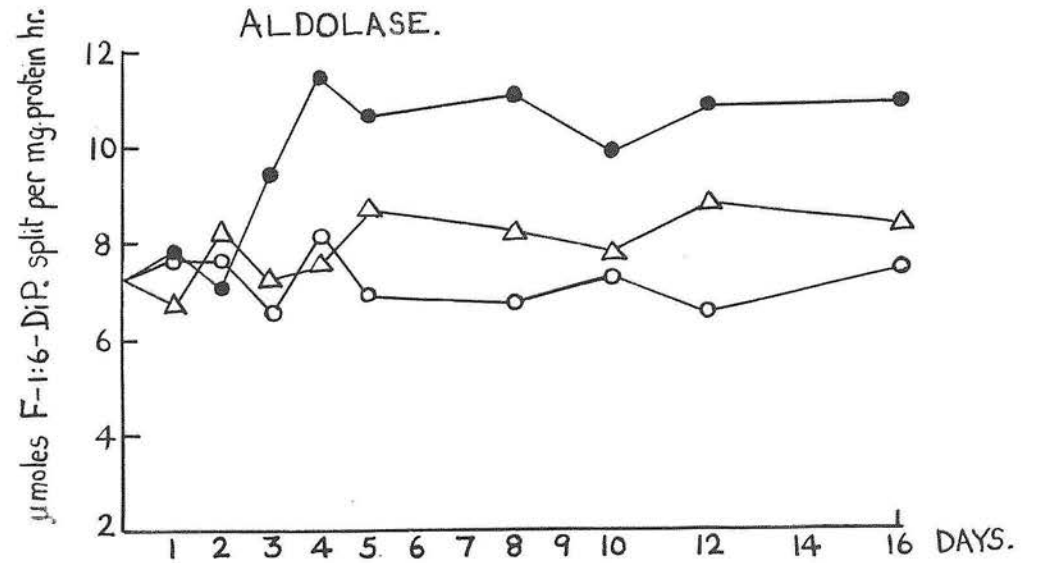
TABLE 12

GROWTH AND GLYCOLYSIS AFTER 35 DAYS EXPOSURE TO 0.10mM DNP

2:4-DNP	-		-		0.1mM		0.1mM	
Prior exposure to 0.1mM	-		35 days		-		35 days	
Days	Protein	QLA	Protein	QLA	Protein	QLA	Protein	QLA
0	288 ± 13		274 ± 11		288 ± 13		274 ± 11	
1	418 ± 16	0.41	400 ± 14	0.37	372 ± 11	0.79	362 ± 11	0.85
2	590 ± 27	0.26	592 ± 17	0.26	438 ± 18	0.87	440 ± 7	1.17
3	813 ± 26	0.25	828 ± 22	0.22	480 ± 10	1.21	473 ± 9	1.48
4	1106 ± 20	0.15	1142 ± 21	0.18	421 ± 14	1.72	440 ± 18	1.63







GRAPH 10

THE EFFECTS OF GROWTH IN 2:4-DNP ON ENZYME ACTIVITIES.

○ No 2:4-DNP ● 0.05mM. 2:4-DNP

△ 0.10mM. ,,

E. THE EFFECTS OF 2:4-DINITROPHENOL ON GROWTH AND METABOLISM UNDER ANAEROBIC CONDITIONS

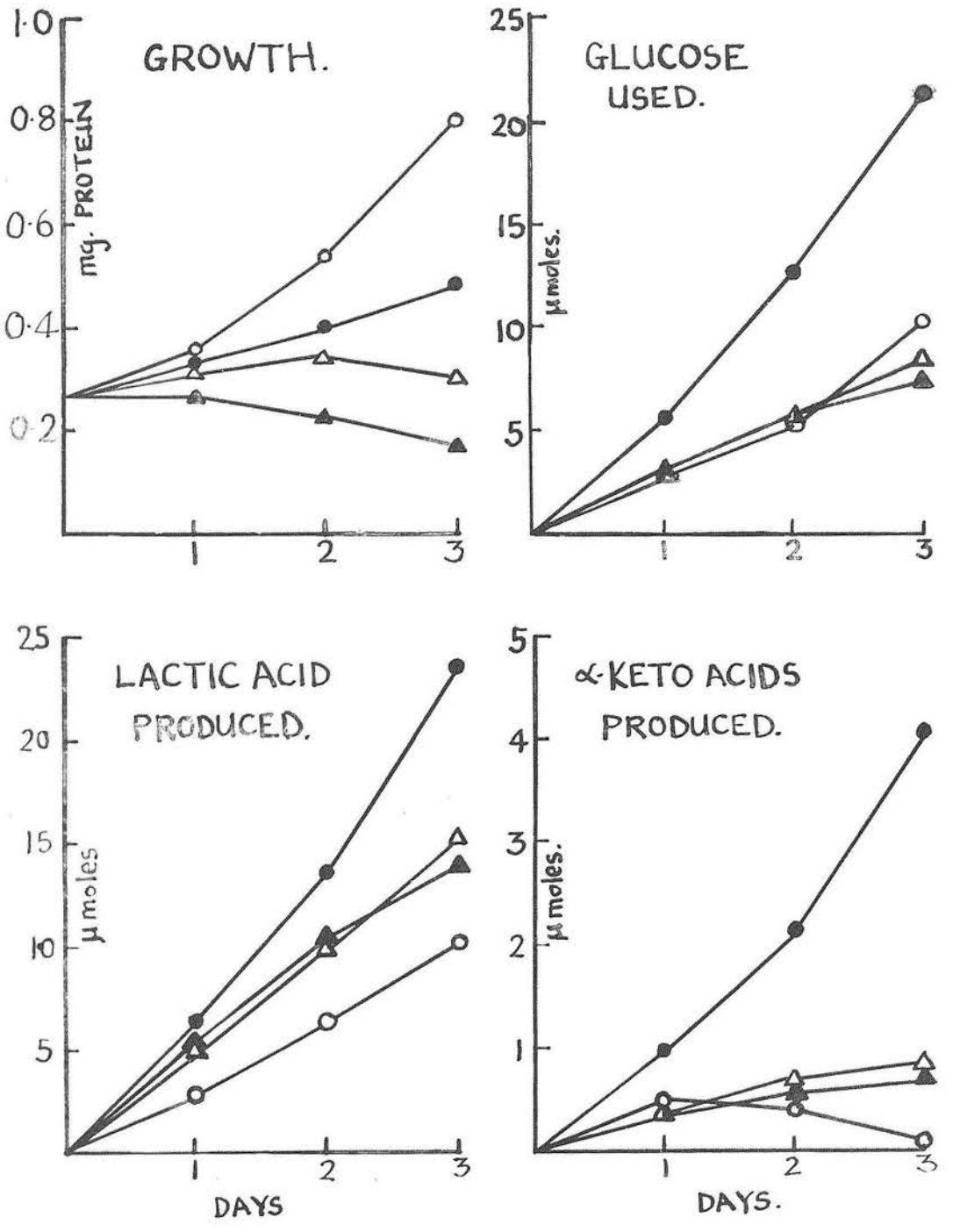
It was consistently found that growth could not be sustained under anaerobic conditions. Typically, however, cell protein increased by about 30% over the first two days subsequent to marked necrosis occurring. Due to the rapid rate at which lactic acid was produced (Graph 11) each experimental culture contained 5.5 ml. medium instead of the usual 4.0 ml. Under these conditions the pH was maintained above 7.0 over 3 days. Tables 13 and 14 show data from two experiments on the effect of 0.10mM 2:4-DNP on anaerobic growth and metabolism. Control cultures under aerobic conditions were included.

Typically the rate of anaerobic glycolysis was 0.6-0.7 u moles lactic acid produced per mg protein hour. Unlike aerobic glycolysis, this did not show any marked decrease over the three days. On the first day anaerobic glycolysis was therefore about twice that of aerobic glycolysis, and on subsequent days this difference increased. In all cases, the rate of anaerobic glycolysis was less than that of 0.10 mM 2:4-DNP stimulated aerobic glycolysis, by from 20 - 38%.

The observable effects of 0.10 mM 2:4-DNP on cells under anoxia were twofold - a marked acceleration of necrosis and a small stimulation of glycolysis. No significant growth was observed when 2:4-DNP was present, although protein maintained itself for twenty-four hours before the onset of necrosis. After 72 hours cultures containing the uncoupler had approximately 40% less cell protein than those without the uncoupler. Anaerobic glycolysis was significantly stimulated by 0.10 mM 2:4-DNP. This increase was small and never greater than 40%. On comparing the six paired observations (3 from each of the two experiments) shown in Tables 13 and 14, this difference is significant with P just under 0.01. The rate of the 2:4-DNP stimulated anaerobic glycolysis was in general comparable to that of 2:4-DNP stimulated aerobic glycolysis at this pH.

The /

The extent to which complete anaerobiosis was achieved by the gassing method used (page 46) is uncertain. The calculations for glucose unaccounted for by glycolysis indicate that little if any oxygen was available to the cells, however the validity of such small "glucose unaccounted for" quotients is questionable due to the standard errors involved. In this respect the appearance of small quantities of α-keto acids in the medium is difficult to explain if complete anaerobiosis prevailed. Assessment of the effects of 2:4-DNP on cells under manometric conditions in which anaerobiosis was more definitely established was carried out later (page 111).



GRAPH 11.

GROWTH AND METABOLISM UNDER ANAEROBIC CONDITIONS WITH & WITHOUT 2:4-DNP.

△ Anaerobic

▲ Anaerobic, 0.1mM. 2:4-DNP.

○ Aerobic

● Aerobic, 0.1mM. 2:4-DNP.

TABLE 13

COMPARISON OF THE EFFECTS OF 1.10mM 2:4-DNP ON GROWTH AND METABOLISM UNDER AEROBIC AND ANAEROBIC CONDITIONS.

	24 hours	48 hours	72 hours
GROWTH (initial: 261 s.d. 12 ug.)			
Aerobic	376 s.d. 14	538 s.d. 17	802 s.d. 23
Aerobic 2:4-DNP	331 13	396 14	480 20
Anaerobic	312 15	342 10	303 18
Anaerobic 2:4-DNP	260 9	222 7	163 16
METABOLIC QUOTIENTS	0-24	24-48	48-72 hours
Glucose used			
Aerobic	0.375	0.320	0.30
Aerobic 2:4-DNP	0.79	0.80	0.85
Anaerobic	0.41	0.33	0.39
Anaerobic 2:4-DNP	0.50	0.45	0.39
Lactic acid produced			
Aerobic	0.37	0.33	0.23
Aerobic 2:4-DNP	0.88	0.86	0.90
Anaerobic	0.73	0.64	0.70
Anaerobic 2:4-DNP	0.86	0.90	0.70
α-keto acids produced			
Aerobic	0.06	0.01	-0.01
Aerobic 2:4-DNP	0.14	0.14	0.19
Anaerobic	0.06	0.03	0.02
Anaerobic 2:4-DNP	0.06	0.03	0.02
Glucose "unaccounted for"			
Aerobic	0.16	0.15	0.18
Aerobic 2:4-DNP	0.29	0.31	0.28
Anaerobic	0.03	-0.01	0.02
Anaerobic 2:4-DNP	0.04	0.00	0.03

TABLE 14

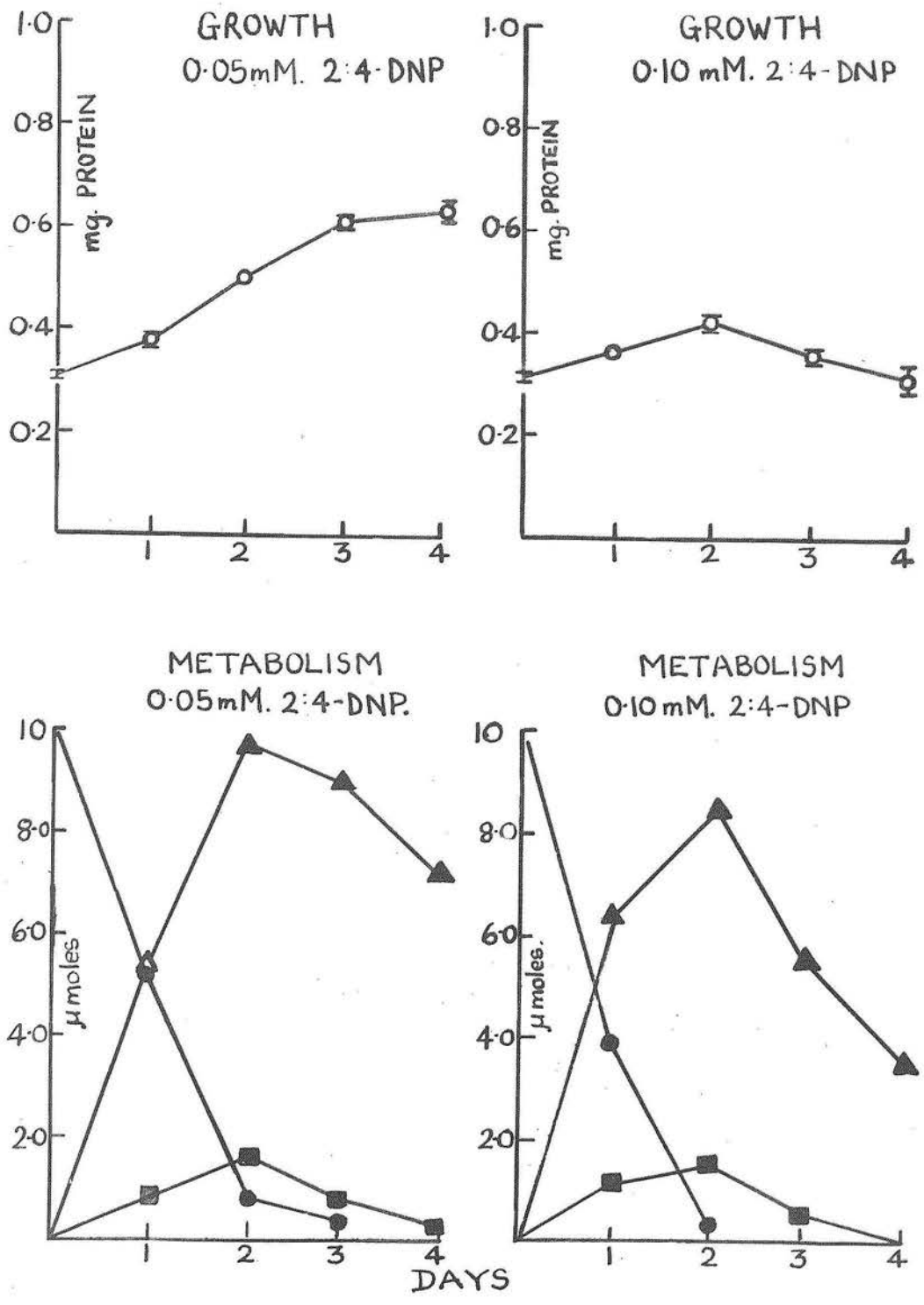
EFFECTS OF 0.10mM 2:4-DNP ON GROWTH AND METABOLISM UNDER AEROBIC AND ANAEROBIC CONDITIONS

	24 hours	48 hours	72 hours
GROWTH (initial: 284 s.d. 13 u.g.)			
Aerobic 2:4-DNP	356 s.d. 9	418 s.d. 16	525 s.d. 20
Anaerobic	349 15	373 12	300 15
Anaerobic 2:4-DNP	298 10	214 8	182 15
METABOLIC QUOTIENTS			
Glucose used			
Aerobic 2:4-DNP	0.81	0.77	0.90
Anaerobic	0.41	0.38	0.30
Anaerobic 2:4-DNP	0.56	0.48	0.35
Lactic acid produced			
Aerobic 2:4-DNP	0.94	0.86	0.98
Anaerobic	0.70	0.68	0.60
Anaerobic	0.99	0.91	0.78
α-keto acids produced			
Aerobic 2:4-DNP	0.13	0.14	0.16
Anaerobic	0.07	0.02	0.02
Anaerobic 2:4-DNP	0.07	0.04	0.01
Glucose "unaccounted for"			
Aerobic 2:4-DNP	0.27	0.27	0.32
Anaerobic	0.04	0.02	-0.02
Anaerobic 2:4-DNP	0.02	0.00	0.00

F. THE EFFECTS OF 2:4-DINITROPHENOL ON CELLS GROWING IN LIMITING CONCENTRATIONS OF GLUCOSE

Previous experiments have shown that cells treated with 2:4-DNP metabolise glucose with the production of lactic acid and α-keto acids into the medium at rates considerably in excess of those of control cells. Further experiments show that on depletion of the glucose in the medium, uncoupled cells significantly utilise the acids produced. The results of an experiment to determine the pattern of metabolism when glucose becomes limiting are shown in Graph 12. In this case the initial glucose concentration was 2.5mM, making a total of 10u moles of glucose available to each culture. At 0.1mM 2:4-DNP the glucose was exhausted within 2 days, and on the third and fourth day more than half of the lactic acid produced into the medium during the first two days was utilised. By the end of the fourth day, all the α-keto acids produced from glucose metabolism had been utilised.

The metabolism induced by 0.05mM uncoupler showed the same general pattern. In this case, however, the utilisation of acids was not as extensive as at the higher 2:4-DNP concentration. The effects of 2:4-DNP on growth and metabolism where pyruvate and lactate are the primary carbon sources are more completely assessed in a later section (Section II).



GRAPH 12.

EFFECTS OF 2:4-DNP ON CELLS GROWING IN LIMITING QUANTITIES OF GLUCOSE.

● Glucose available; ▲ Lactate in medium; ■ α-keto acids in medium:

G. GROWTH AND METABOLISM IN SODIUM SALICYLATE

Experiments were undertaken to assess the effects of sodium salicylate on growth and metabolism, using essentially the same experimental procedure as that for 2:4-DNP. Preliminary experiments indicated that 1.0mM salicylate produced no significant difference in cell growth and metabolism over three days and no further investigations were made at this concentration. Graph 13 and Table 15 show the results of a typical experiment in which concentrations of 2mM, 4mM and 10mM sodium salicylate were used.

Like 2:4-DNP, sodium salicylate produced a marked effect on growth. At the highest concentration used (10mM) growth was completely inhibited, and extensive necrosis occurred. In the case of 4mM, cell protein increased approximately two-fold over the four days compared with an over four-fold increase in the control cultures, and the increase was linear during this time. At 2mM, salicylate growth was exponential with a mean generation time of 60 hours compared with 46 hours for the control cultures and where growth was allowed to proceed to stationary phase, there was no significant difference in the final cell protein values attained.

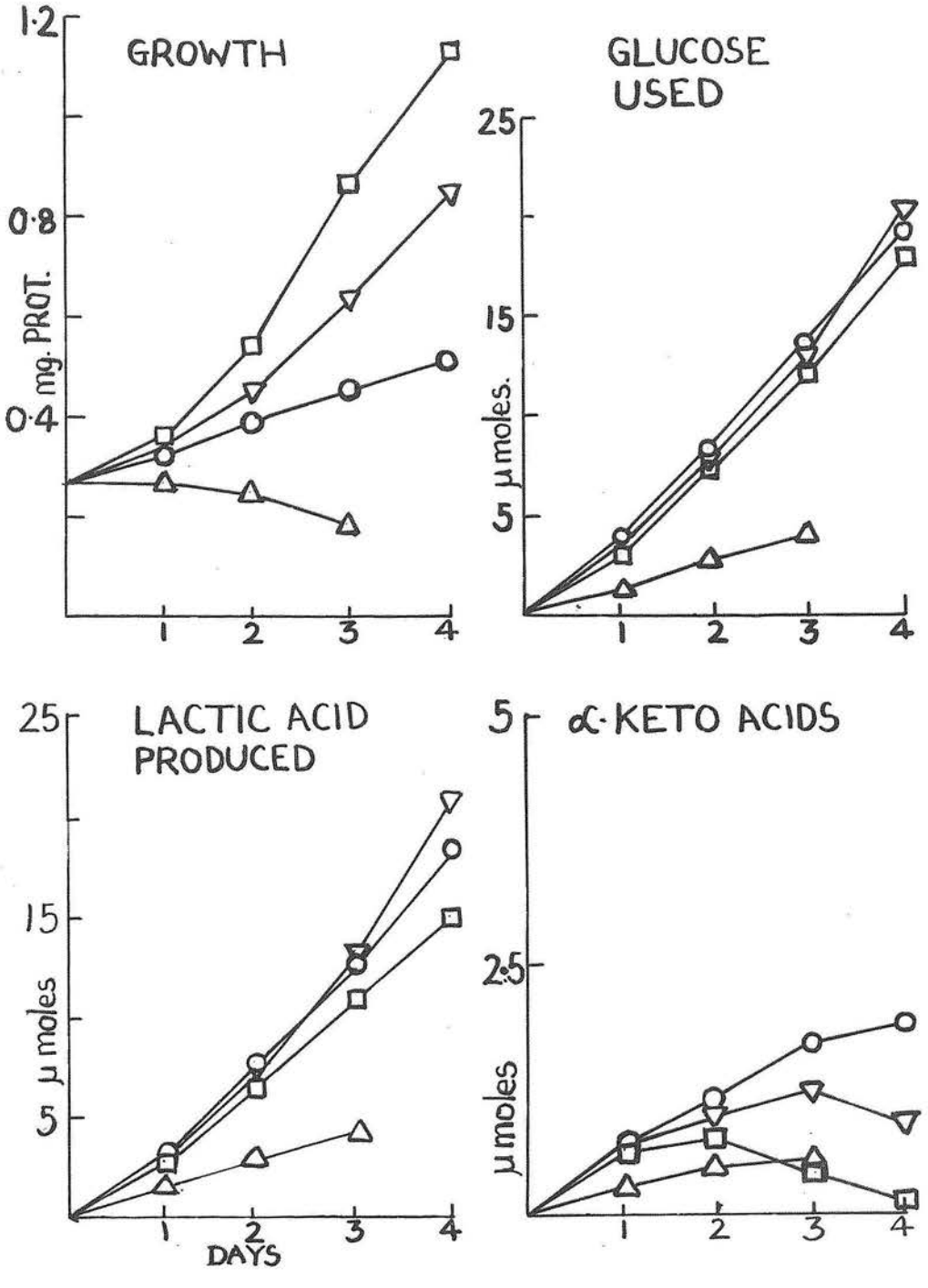
Although the effects of the three concentrations of salicylate tested were comparable to some extent with the effects of the three concentrations of 2:4-DNP tested, (0.15, 0.10 and 0.05mM) relative to growth, the effects on metabolism were much less marked. At 10mM salicylate, the quotients for glucose used and lactic acid produced were of the same order as those of the control cultures and did not show any obvious trend with time. α-keto acids were not produced into the medium during the first day as rapidly as in the control and other experimental cultures, and on subsequent days the rate of production decreased further. From the first day the quotients for glucose "unaccounted for" were considerably lower than those of the controls. The validity of metabolic quotients for cultures in 10mM salicylate is, of course, subject to the same criticisms as were applied to quotients calculated for

0.15mM 2:4-DNP treated cells (page 81) with respect to their necrotic state. The quotients for glucose used and lactate produced by 4mM salicylate treated cells were each in the region of 0.5 u moles per mg. cell protein hour, and again no significant trend appeared from day to day. Those quotients were about 20% higher than those for the controls over the first day, and this difference increased considerably with the characteristic fall in the respective control quotients during subsequent days. In contrast to the situation with 0.10mM 2:4-DNP where the quotients for glucose and lactate were respectively five and eight or more times those of the controls on the last day of the experiment, the quotients for 4mM salicylate treated cells were merely two and three times the relevant control quotients at the same period. The rate of α-keto acid production into the medium was similarly much lower than in 0.1mM 2:4-DNP treated cells, although significantly higher than that in control cultures. Although there was no net decrease in α-keto acids in the medium, the rate of production per mg. cell protein decreased considerably during the experiment, and on the fourth day was only 20% of the rate on the first day. The quotients for "glucose unaccounted for" induced by 4mM salicylate treatment were in general about 25% higher than those for the control cultures, and showed no significant variation from day to day.

At the lowest salicylate concentration tested (2mM) the quotients for glucose used and lactic acid produced were possibly slightly higher than those of the controls, and remained relatively constant during the experiment. The results showed a slight decrease in the α-keto acids in the medium during the fourth day. The quotients for "glucose unaccounted for" were essentially the same as those of the control cultures.

As shown in Graph 13 the lactic and α-keto acids produced into the media during those experiments never reached concentrations comparable with those in the previous experiments with 2:4-DNP. One result of this was that the pH of the media did not fall to the same extent, and even in the case of

4mM salicylate the pH did not fall below 7.1 in the course of the experiments.



GRAPH 13

GROWTH AND METABOLISM IN SODIUM SALICYLATE.

□ No salicylate ▽ 2mM. salicylate

○ 4mM. salicylate △ 10mM. salicylate.

TABLE 15

THE EFFECTS OF SALICYLATE ON METABOLIC QUOTIENTS

(Growth depicted in Graph 13).

Hours	0 - 24	24 - 48	48 - 72	72 - 96
GLUCOSE USED				
CONTROL	0.40	0.38	0.29	0.24
2mM	0.46	0.46	0.41	0.41
4mM	0.52	0.54	0.52	0.48
10mM	0.27	0.29	0.19	-
LACTIC ACID PRODUCED				
CONTROL	0.38	0.35	0.25	0.17
2mM	0.42	0.47	0.37	0.44
4mM	0.46	0.51	0.51	0.48
10mM	0.28	0.24	0.23	-
α-KETO ACIDS PRODUCED				
CONTROL	0.08	0.01	-0.02	-0.01
2mM	0.10	0.03	0.02	-0.02
4mM	0.10	0.05	0.06	0.02
10mM	0.05	0.05	0.02	-
GLUCOSE "UNACCOUNTED FOR"				
CONTROL	0.17	0.20	0.18	0.16
2mM	0.20	0.21	0.19	0.19
4mM	0.24	0.25	0.23	0.24
10mM	0.11	0.14	0.07	-

MANOMETRIC EXPERIMENTS

A. PRELIMINARY EXPERIMENTS

(i) Endogenous Respiration

Preliminary experiments revealed that over the first 1 - 1½ hours after the transfer of cells from the growth medium to manometers, the endogenous respiration rate fell from an initial QO_2 (protein) of 17-21 to a QO_2 of 10-13. This latter rate remained more or less stable for the next 2½-3 hours, before a further decrease accompanied by increasing cell necrosis became apparent. Addition of glucose (10mM) made little if any difference to the initial endogenous rate but in contrast, the rate with glucose (QO_2 17-21) remained constant for up to 4½ hours.

The marked fall in endogenous respiration rate during the first 1-1½ hours is unlikely to be a result of cell necrosis during this period, for glucose added after 1½ hours, increased respiration to a rate similar to that of cells which had received glucose at the commencement. Graph 14 is typical. The most likely explanation is that it is due to the rapid depletion of a pool of readily oxidisable substrates. The nature of the substrates for endogenous respiration was not thoroughly investigated, but the following points are relevant. No qualitative or gross quantitative differences in the free amino acid pool within the cells before and after up to three hours endogenous respiration were found on chromatographing extracts in phenol/water and spraying with ninhydrin. This indication that amino acids were not the main substrates at least, was supported by the finding that the total α-amino content of the cell pool was not significantly reduced from 0.242 s.d. 0.015 u moles per mg. cell protein during endogenous respiration.

The finding that considerable quantities of lactic acid are produced from endogenous substrate under anaerobic conditions (page 112) suggests that glycogen is the main endogenous substrate. Calculations on the extent of endogenous

respiration, and anaerobic endogenous glycolysis, indicate that these could be accounted for if glycogen comprised about 2% of the total dry weight of the cells. Attempts to isolate glycogen from cells equivalent to 50 mg. protein by the method described on page 69 were, however, inconclusive. The matter was not pursued further as it became clear that very large quantities of cells were necessary before meaningful results could be obtained. The respiratory quotient of endogenous metabolism was measured and consistently found to be between 0.85 and 0.92, with no significant difference between the first hour and later phases. Since conclusions on the nature of the substrate from RQ values are hazardous (Umbreit, Burris and Stauffer, 1957), these values are included merely as an index of metabolism under the stated conditions.

Although the nature of the endogenous substrate was not completely resolved, preincubation of cells in Krebs Ringer Phosphate for 1½ hours before experiments was routinely carried out for practical reasons.

(ii) Glucose concentration

Graph 15 shows that at pH 7.4, respiration rate increased with external glucose concentration to about 3mM, and remained constant to 40mM. Lactic acid production appeared to reach maximum rate at a rather higher external glucose concentration (5mM) thereafter maintaining this at concentrations up to 40mM also. These findings differ from those of Danes and Paul (1961) who observed a marked depression of L cell respiration by glucose concentrations greater than 22mM. As this was most obvious at high respiratory rates, its absence from these experiments may be due to the fact that in general the QO_2 was considerably lower than that measured by Danes and Paul. A significant reduction in respiration rate was in fact, obtained with 60mM glucose on two occasions but this was uncharacteristic of a Crabtree Effect as the rate of glycolysis decreased also, suggesting an adverse effect of high glucose or low NaCl concentration on the cells.

(iii) Effects of cell concentration on metabolism

As cell concentration is known to influence metabolism (Danes and Paul, 1961), experiments were carried out to assess this in the present system. The results of two experiments are shown in Table 16 and show that respiration rates were not affected by quantities of tissue between 3 and 11 mg. protein per manometer. The rate of glycolysis was relatively constant between 6 and 11 mg. protein per manometer, at about 0.4 u moles lactic acid per mg. per hour, but with smaller quantities of tissue per manometer, the rate of glycolysis increased. At 3 mg. protein per manometer, the rate of glycolysis approximately doubled.

(iv) Effects of pH on metabolism and assimilation

Although several authors have shown that pH markedly influences glucose metabolism and growth (page 32), the effects of pH on assimilation under short term manometric conditions have not been characterised. It is in fact, difficult to obtain significant results on the extent to which unlabelled glucose is assimilated by mammalian cells at different pH values for several reasons. The most obvious of these is that assimilation cannot be measured directly, but must be calculated by subtraction of the glucose glycolysed and the glucose oxidised from the total glucose used. This means that sufficient glucose must be assimilated to give meaningful results, taking into account the standard errors of the methods used for determining these quantities. In addition, this must be achieved before the production of lactic acid at high pH has significantly altered the pH of the medium. A further difficulty is involved in the calculation of the glucose oxidised, particularly acute when the endogenous respiration is relatively high as in most mammalian cells and when the extent to which exogenous substrates suppress this is unknown. The latter point has been discussed at length by Van Niel (1943) Moses and Syrett (1955) and others, and the only valid conclusion is that no generalisations can be made, the situation

varying from tissue to tissue. A further consideration is that small quantities of glucose carbon may be lost from the cell as organic or amino acids, thereby decreasing the accuracy of the calculation for glucose assimilated.

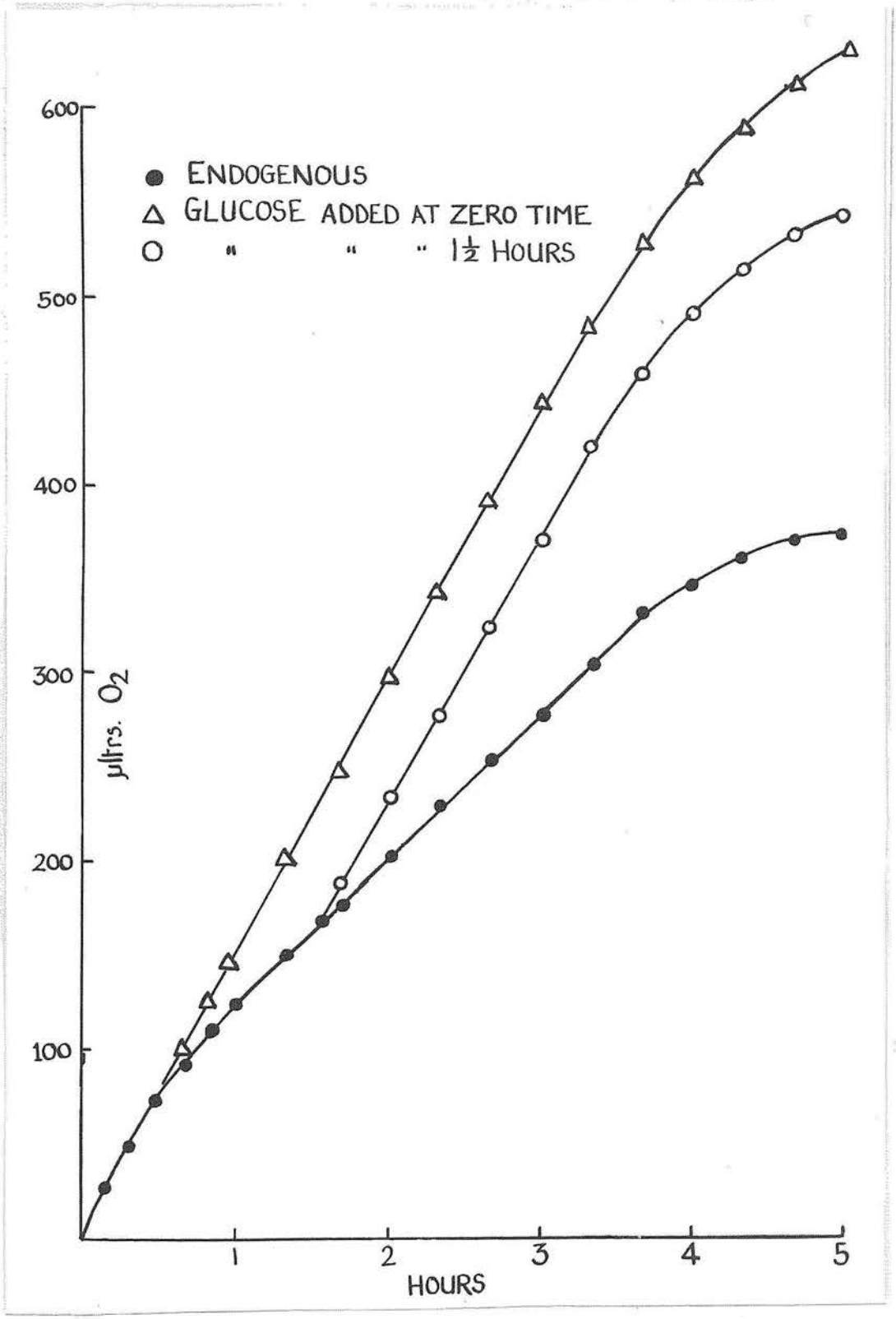
In the course of several experiments, a definite trend was observed for glucose used unaccounted for by glycolysis and oxidation, to be greater at the more physiological pH levels than at 6.5 and 7.9. Table 17 shows data from two such experiments. For convenience, the assumption has been made that endogenous respiration is not influenced by addition of glucose. This trend was confirmed by an experiment using 14-C glucose (Table 18) in which the 14-C incorporated into acid precipitable cell material during 1 hour at pH 7.4 was in the region of 1.7 times that at pH 6.5 and 7.9. These are probably more significant figures than those for total intracellular 14-C, as the 14-C in the acid soluble fraction very likely reflects differences in glycolytic rate to some extent at least.

It is of note that the total 14-C assimilated was in all cases less by 50% or more than that calculated on the assumption that endogenous respiration is unaffected by the presence of glucose. Since the activity of the CO_2 evolved was not estimated, it is not possible to decide from those results, whether this was due to the suppression of endogenous respiration by exogenous substrate, or the loss of 14-C labelled material such as Citric Acid Cycle intermediates from the cells. That the first of these is operating to some extent at least is indicated insofar as later experiments (Table 18b) show that considerably more 14-C appears in the CO_2 than expected if endogenous respiration is not suppressed. Medes and Weinhouse (1958) and Emmelot and Nout (1959) have shown that glucose 14-C readily replaces endogenous carbon in the production of carbon dioxide by ascites tumour cells. From Table 18 it is clear that, if the difference between the calculated and the actual glucose assimilated was in fact oxidised, endogenous respiration in L cells under these conditions would be about 40% suppressed by addition of glucose.

No significant difference was found in respiration rates at pH 6.9, 7.4 and 7.9. Typically the rate at pH 6.5 was lower, but never by more than 20 per cent of the average rate at the higher pH values. Over the range 6.5 to 7.9 therefore, pH did not markedly affect respiration.

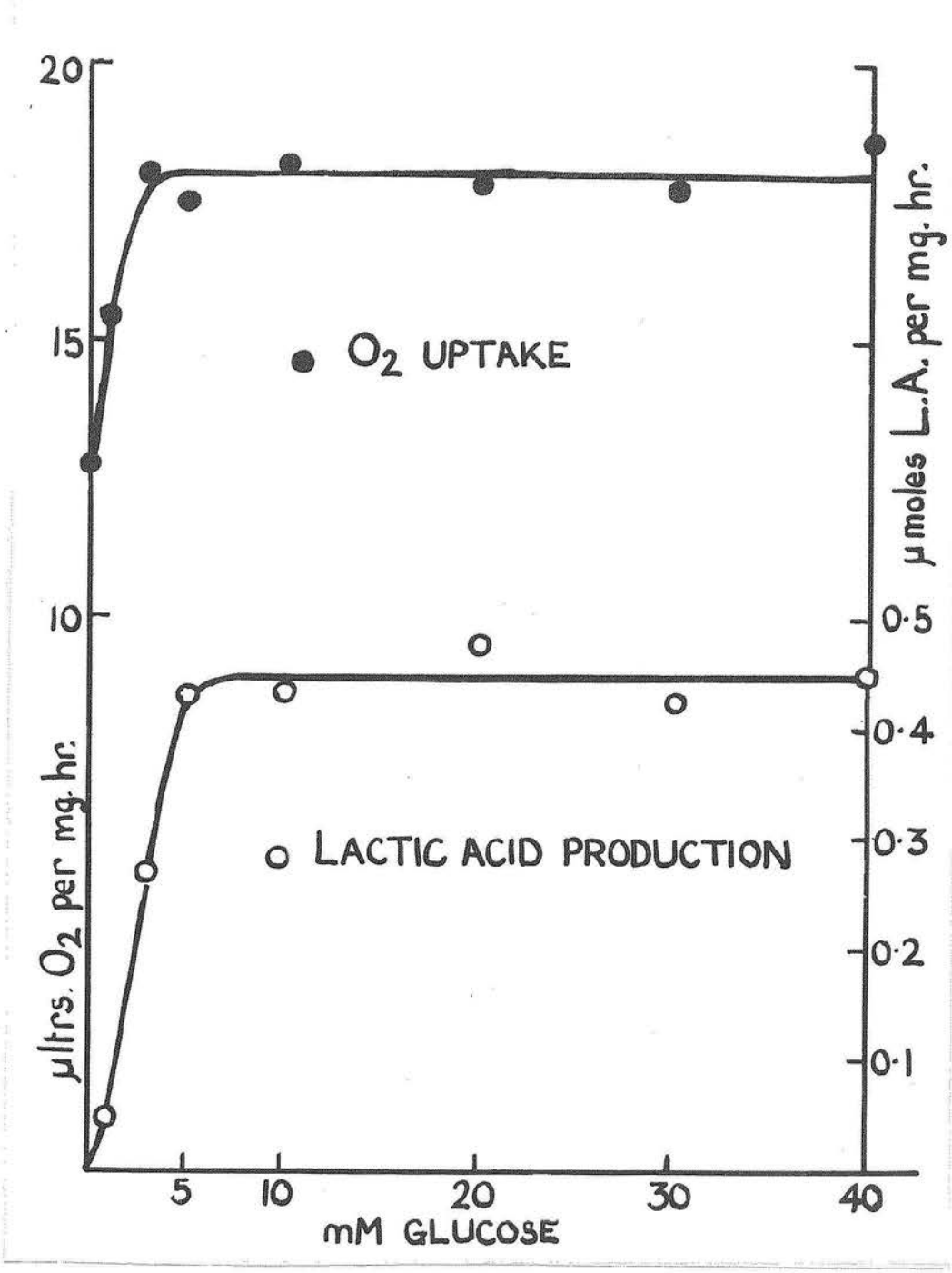
Glycolysis, however, was profoundly influenced by pH. At 6.5 virtually no lactic acid was produced, and at 6.9 never more than 0.09 u moles per mg. hour. In contrast to this, the rate at 7.4 was 0.3-0.4 u moles per mg. hour, and at 7.9 about 0.8 u moles. Glycolysis therefore increased approximately 9 times between pH 6.9 and pH 7.9.

The production of α-keto acids showed a similar trend with increasing pH, but when present, these were found at much lower concentrations than lactic acid, and their occurrence in the medium was much less predictable.



GRAPH 14

Manometric experiment over 5 hours
with and without glucose.



GRAPH 15.

THE EFFECTS OF GLUCOSE CONCENTRATION ON THE RESPIRATION RATE AND RATE OF LACTATE PRODUCTION.

TABLE 16

INFLUENCE OF CELL CONCENTRATION ON RESPIRATION AND GLYCOLYSIS IN KREBS RINGER PHOSPHATE pH 7.4

EXPERIMENT 1 (2 hours)

mg. protein per flask	Endog. QO_2	Exog. QO_2	L.A. prod. mg. in 2 hrs.	Total L.A. produced
3.31	12.0	19.7	1.6	5.28
6.40	12.4	20.3	0.98	6.25
8.78	12.5	20.6	0.82	7.20
11.22	12.1	20.2	0.72	8.08

EXPERIMENT 2 (1 hour)

Mg. protein per flask	Endog. QO_2	Exog. QO_2	L.A. prod. mg./hour	Total L.A. produced
2.93	13.1	18.5	0.73	2.14
6.20	12.8	17.9	0.43	2.68
8.57	12.6	18.9	0.38	3.26
11.04	13.0	19.1	0.44	4.85

TABLE 17

EFFECTS OF pH ON GLUCOSE METABOLISM (2 hours)

	Exp.1	7.2mg. protein			Exp.2	8.1mg. protein		
Initial pH	6.5	6.9	7.4	7.9	6.5	6.9	7.4	7.9
Final pH	6.7	6.9	7.2	7.65	6.5	7.0	7.3	7.6
QO_2 (Exogenous)	17.2	19.0	19.1	18.9	15.5	19.4	18.5	18.5
QO_2 (Endogenous)	11.6	12.5	12.0	13.2	9.9	11.4	11.1	11.3
Q lactic acid	0.03	0.09	0.33	0.81	0.00	0.08	0.35	0.84
Glucose used	2.26	2.95	4.77	7.74	1.84	3.34	5.52	9.16
Lactic acid produced	0.48	1.28	4.76	11.60	-	1.30	5.62	13.53
α -Keto acids produced.	0.12	-	0.48	0.76	-	-	0.15	0.22
Glucose oxidised	0.60	0.69	0.71	0.53	0.66	0.97	0.89	0.85
Glucose unaccounted for	1.36	1.62	1.43	1.03	1.18	1.72	1.75	1.44
ATP produced from glucose metabolised to acids	0.96	1.28	6.68	14.64	-	1.30	6.22	14.41
ATP produced from glucose completely oxidised	70.	77	75.	73	70.	88.	84.	84.
Total ATP synthesised	71.	78	82	88	70	89	90	98

TABLE 18

EFFECTS OF pH ON 14-C GLUCOSE METABOLISM

(1 hour)

Initial pH	6.5		7.4		7.9	
Exogenous respiration	142	170	173	165	163	175
Endogenous respiration	112	(112)	110	(110)	110	(110)
Glucose oxidised	0.22	0.43	0.47	0.41	0.41	0.45
Lactic acid produced	0.0	0.21	2.70	2.85	7.36	8.04
α-keto acids produced	0.0	0.06	0.15	0.21	0.27	0.42
Glucose accounted for	0.22	0.57	1.89	1.94	4.22	4.68
Glucose used	1.53	1.20	2.61	2.84	4.85	5.28
Glucose unaccounted for	1.31	0.63	0.72	0.90	0.63	0.60
14-C glucose (1 u mole: 2921 cpm)						
cpm in acid-soluble frac.	235	212	125	241	200	181
u moles " " " "	0.08	0.07	0.04	0.08	0.07	0.06
cpm in acid-ppt. fraction	478	551	971	1018	527	632
u moles " " " "	0.16	0.19	0.33	0.35	0.18	0.22
Total 14-C glucose incorporated	0.24	0.26	0.37	0.43	0.25	0.28
Glucose unaccounted for less 14-C glucose incorporated	1.07	0.37	0.35	0.47	0.38	0.32
Depression of endogenous respiration by glucose, assuming that this is respired		45	42	56	47	39

9.3 m.g. protein per flask

TABLE 18b

SUPPRESSION OF ENDOGENOUS RESPIRATION BY ADDITION OF 14-C GLUCOSE

	Flask 1		Flask 2	
		counts per 100 secs.		counts per 100 secs.
Exogenous respiration (u ltrs.)	182		169	
Endogenous respiration (u ltrs.)	104		104	
Glucose used (u moles)	2.92	7835	2.86	7687
Glucose used for acid production	1.42	3831	1.53	4113
Glucose converted to CO_2 assuming that endogenous respiration is unaffected by glucose	0.58		0.48	
Glucose assimilated (calculated)	0.92		0.85	
Glucose converted to CO_2 (measured)	0.91	2422	0.67	1815
Glucose assimilated (measured)	0.52	1402	0.50	1350
Percentage suppression of endogenous respiration by glucose	42%		24%	

Experiment over 1 hour. 8.4 mg. protein per flask. Specific activity of glucose 1 u mole = 2,682 c.p. 100 secs.

B. EFFECTS OF 2:4-DNP AND SODIUM SALICYLATE ON RESPIRATION AND GLYCOLYSIS

(i) Effects of 2:4-DNP on respiration

Addition of 2:4-DNP greatly influenced the respiration rates of cells metabolising glucose. Graph 16 shows the results of an experiment on the effects of various concentrations of the uncoupler over 2½ hours. The results are typical.

At 0.01mM 2:4-DNP a small stimulation of respiration rate amounting to 5-15% was consistently observed. At 0.05mM this increased to between 100 and 120%, and as with 0.01mM, the rate remained constant for 2½ hours or longer. Maximum stimulation of respiration was induced by about 0.07mM 2:4-DNP, the stimulation in this case being up to 150% of the control rate. Typically the rate remained constant for 1½-2 hours and thereafter decreased until at 2½ hours it was about 40% of the initial rate.

At higher concentrations, the initial respiratory rates became progressively lower, and the onset of inhibition more rapid. For example, at 0.10mM, the initial rate, invariably the same as that at 0.05mM, began to fall after only 1-1½ hours and by 2½ hours had decreased to about 25% of its initial value. At 0.20mM this trend was even more apparent. In several experiments high concentrations of 2:4-DNP were investigated. At 1.0 and 2.0mM, the initial rates were 70% and 50% of the controls without 2:4-DNP. Over 2½ hours those rates decreased by about 40%, but were never completely suppressed even after another hour. It would appear that very large concentrations of the uncoupler are required to inhibit respiration completely. This aspect was not pursued further.

Graph 17 shows the results of an experiment on the effects of DNP on endogenous respiration. This experiment is complementary to that of Graph 16 already discussed, both experiments being done on the same day with cells from the same culture. At all concentrations of 2:4-DNP tested, an initial

stimulation of endogenous respiration was observed. At 0.01 mM this was small, and within about 20 minutes decreased to a rate comparable with the control without the uncoupler ($Q0_2$:10). This rate was maintained for up to 2½ hours. Concentrations of 0.05, 0.07 and 0.10mM 2:4-DNP produced marked initial stimulations, the relative initial $Q0_2$ values being about 20, 26 and 26. These initial rates were rapidly followed by severe inhibition reducing them to a relatively constant $Q0_2$ of about 2.8 for each concentration. This became evident within 20 minutes in the case of 0.07 and 0.10mM, and 30 minutes with 0.05mM. At 0.20mM, 2:4-DNP the initial rate ($Q0_2$:15) was less than at the lower concentrations tested, and very rapidly decreased to about 2.8 also. A point of some interest therefore, is that after the initial stimulation, the final respiratory rate for each concentration of 2:4-DNP between 0.05 and 0.10mM was stable and similar, with a $Q0_2$ of about 2.8 compared with 10 for the control without 2:4-DNP. This has been observed on several occasions, the $Q0_2$ always stabilising between 2.5 and 3.5.

It was important to determine whether the observed inhibitory phase of 2:4-DNP action on respiration was the result of extensive cell damage or was a general depression of respiration. The problem of defining and determining cell death has already been discussed (page 51). Table 19 shows that 2½ hours under manometric conditions did not cause marked necrosis in the control cells with and without glucose, the percentage of cells staining with dye in both cases being between 6 and 8, compared with an initial count of about 4%. 2:4-DNP significantly increased necrosis in endogenous and exogenous experiments. Even at the highest concentration used, however, (0.2mM), a maximum of 16% stained and there was no significant difference between cells using endogenous and exogenous substrate. Little significance can be attached to this similarity between 2:4-DNP induced cell necrosis under exogenous and endogenous conditions of metabolism, as the action of the uncoupler in the former situation was accentuated by falling pH due to increased glycolysis (page 76).

The general reliability of the sulphonated dye was supported by investigation into the ability of washed cells to reduce tetrazolium dye under nitrogen. About 80% of the 0.2mM treated cells showed reductive activity to some extent.

The data from these experiments, therefore, indicates that while necrosis of a proportion of cells may contribute to the inhibition of respiration induced by 2:4-DNP, this is insufficient to be the primary cause.

Cells treated with 2:4-DNP before exogenous substrate is added never attain the respiratory rates of cells which have received the substrate and uncoupler simultaneously. The results of an experiment where glucose was added 0, 20, 40 and 80 minutes after 0.05mM 2:4-DNP are shown in Graph 18. This concentration of uncoupler was used, as it is the greatest concentration which induces a high and constant respiratory rate with glucose, without subsequent inhibition. The effect of the uncoupler on endogenous respiration in this case was similar to that already observed, with the rate falling in the first 75 minutes from an initial $Q0_2$ of 22 to a steady final $Q0_2$ of 3.3. Addition of glucose at 0, 20, 40 and 80 minutes substantially increased the $Q0_2$ values at these points. The new rates varied with the time the cells had been exposed to 2:4-DNP before the addition of glucose. The respective rates obtained on adding glucose 0, 20, 40 and 80 minutes after the DNP were 38.5, 23, 15 and 8.7. The respective endogenous rates at these points were estimated as 22, 10, 7 and 3.3. To some extent, therefore, respiratory rates obtained on adding glucose reflected the 2:4-DNP induced endogenous rate at the time of addition.

Estimation of dead cells at the end of the experiment indicated that about 15% of the cells were necrotic in those flasks to which glucose was added after 40 and 80 minutes. Only about half of this number were necrotic where the glucose was added at the same time as the uncoupler. The data relevant to this and a similar experiment is shown in Table 20.

(ii) Effects of 2:4-DNP on aerobic glycolysis

Analyses of the medium of cells metabolising glucose in the presence of 2:4-DNP showed that the uncoupler greatly increased the rate of lactic acid production. Table 19 contains data from the experiment shown in Graphs 16 and 17. The lactic acid present at the higher 2:4-DNP concentrations (0.1 and 0.2mM) at the end of the experiments (2½ hours) was approximately six times greater than that of the control. This cannot, of course, be taken as an index of rate of production. Low pH values (6.4 - 6.5) and high rates of glucose utilisation were associated with the high concentrations of lactic acid found in the media of cells metabolising in the presence of the uncoupler. Glucose unaccounted for on the assumptions discussed in page 104 showed a definite trend to decrease at 0.1mM 2:4-DNP and higher concentrations. This was noted on several occasions, but the results of such calculations were often erratic and have not been analysed since reliable results on the effects of 2:4-DNP on the incorporation of 14-C glucose were later obtained (page 114). The results of a series of experiments on the stimulatory effects of 2:4-DNP on glycolysis are presented in the next section.

(iii) Modification of the effect of 2:4-DNP by pH.

It has long been known that the activity of weak acids on biological material is influenced by pH. The foregoing experiments showed that 2:4-DNP stimulates glycolysis to an extent which alters the medium pH considerably under both growth and manometric conditions. It is also of significance that inhibition of glucose respiration was most marked at those concentrations of 2:4-DNP which produced the greatest decrease in pH. That other factors are operating in the inhibition of respiration is, of course, obvious from the effects of 2:4-DNP on endogenous respiration and on exogenous respiration at very high concentration.

Experiments were undertaken at pH 6.6, 7.4 and 7.9 to determine the importance of pH in the action of 2:4-DNP. These were terminated after 30 minutes to prevent modification of the pH of the medium by metabolism and because preliminary experiments showed that the rate of 2:4-DNP stimulated glycolysis was seldom constant for more than one hour. The lactic acid found in the medium after 30 minutes was therefore taken as being proportional to the rate of production. The results are shown in Graphs 19 and 20. The points for the pH 6.6 and 7.9 Graphs are means of two experiments, the actual deviations being shown. The Graphs for pH 7.4 were constructed from 6 experiments, some being done for other reasons, and in this case standard deviations are shown.

Graph 19 shows that the essential features of 2:4-DNP action on respiration at pH 6.6 and 7.9 were similar to those already noted at pH 7.4; namely that with increasing concentrations of 2:4-DNP, respiration was first stimulated and then inhibited. The action of 2:4-DNP on these processes was, however, greatly modified by pH. For example, although approximately similar maximum respiratory rates were attained at pH 6.6, 7.4 and 7.9, these were induced by 2:4-DNP concentrations of 0.03 - 0.04, 0.075 and 0.125mM respectively. At pH 7.9, therefore, 3 - 4 times more 2:4-DNP than at pH6.6 was required to produce the same result. The results signify that a fall in the pH of the medium of cells metabolising glucose in 2:4-DNP concentrations greater than 0.07mM would be accompanied by a depression of respiration rate. In contrast, a fall in pH at 0.05mM would have much less effect on respiration, and at 0.01 mM might be expected to produce a small stimulation.

Graph 20 shows that a somewhat similar pH dependant relationship holds for the actions of 2:4-DNP on glycolysis. At pH 7.4 the maximum rate of glycolysis was induced by 0.20mM and was over 5 times the rate of the control. At pH 6.6 the maximum rate was induced by about 0.08 mM, less than half the concentration required at pH 7.4. At pH 7.9, 0.30mM 2:4-DNP was

required to produce a similar effect. The results again show that a change in the pH of the medium as a result of the cells own metabolism would considerably modify the action of the uncoupler. For example, at concentrations of 2:4-DNP between 0.05 and 0.10mM, a fall in the pH from 7.4 to 6.6 would result in increased glycolysis, and at 0.15 mM and over, such a fall would result in progressive inhibition of glycolysis.

Comparison of Graphs 19 and 20 shows that considerably greater concentrations of 2:4-DNP were required to induce maximal glycolysis than maximal respiration. At each pH the maximum glycolytic rate was induced at a concentration which exerted an inhibitory effect on respiration. At pH 7.4 for instance, maximum glycolysis was induced by 0.2mM uncoupler, at which concentration respiration rate was only 65 - 70% of its maximum. At each pH studied, the concentration of uncoupler required for maximum glycolysis was at least twice that required to induce maximum respiration rate.

(iv) Effects of 2:4-DNP on Anaerobic Glycolysis

Initial experiments indicated that, although L cells showed a marked Pasteur effect under nitrogen, the extent of anaerobic glycolysis never reached that of the maximum 2:4-DNP stimulated aerobic glycolysis. Experiments were conducted at pH 7.4 to analyse this further. The results are shown in Table 21.

Anaerobic glycolysis was high, the average Pasteur effect over 5 experiments being 3.3 (s.d. 0.5). On average the rate of 0.2mM 2:4-DNP stimulated aerobic glycolysis was 1.7 (s.d. 0.2) times that of anaerobic glycolysis. The effect of 2:4-DNP on anaerobic glycolysis was assessed at 0.10, 0.15 and 0.20mM concentrations. Maximum stimulation appeared to be induced at about 0.15mM, but this was small and variable. Over 6 experiments, the 2:4-DNP induced rate was on average 1.3 (s.d. 0.18) times that of the controls. Analysis of the results by comparison of paired observations shows that this is significant at the 0.01 level. In every experiment the maximum rate of 2:4-DNP induced

aerobic glycolysis was greater than that of 2:4-DNP induced anaerobic glycolysis.

(v) Effects of 2:4-DNP on endogenous metabolism under anaerobic conditions

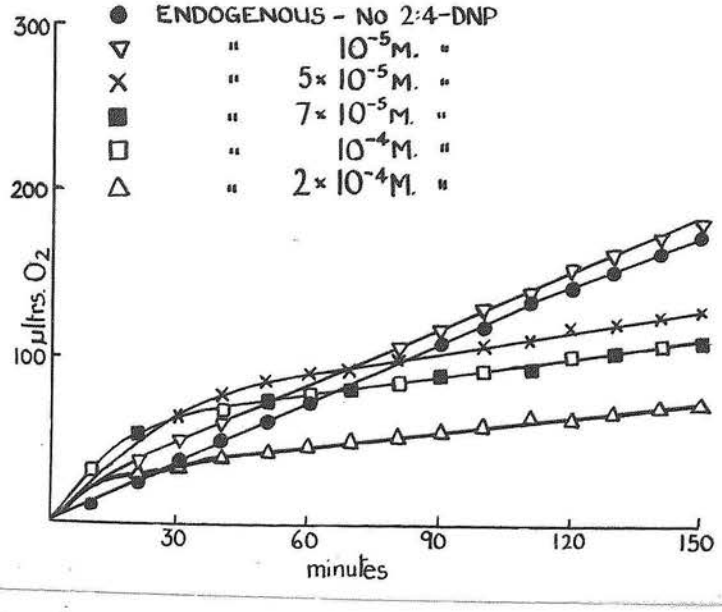
Experiments were carried out on cells freshly transferred from growth medium, to determine the extent to which anaerobic glycolysis of endogenous substrate occurred, and the effects of 2:4-DNP on this. Manometer flasks containing washed cells suspended in KRP at pH 7.4 were nitrogen gassed for 30 minutes while shaking. After a further ten minutes, the sidebulbs containing 2:4-DNP or KRP were tipped. At this point, flasks were sacrificed by addition of acid, and used to give a measurement of the lactic acid produced during the period before tipping. One hour or more after tipping, experiments were terminated by the addition of acid to each flask. The results are presented in Table 22 and show that a considerable quantity of lactic acid was produced from endogenous substrate. Acid production, however, fell off relatively quickly and Experiment 3 of Table 22 shows that within 2½ hours after the commencement of nitrogen gassing, it was negligible. It is of some relevance that the quantity of acid produced during this time could be accounted for on the assumption that glycogen is present to about 2% of the dry weight of the cell. It is clear too that the oxidation of substrate equivalent to the lactic acid produced could substantially account for the endogenous respiration already commented upon.

Anaerobic endogenous glycolysis was severely inhibited by 2:4-DNP. At 0.10mM uncoupler, the inhibition was as great as 70 - 75%, and even 0.01mM inhibited lactic acid production by 20 - 35%.

(vi) Effects of Sodium salicylate on respiration and glycolysis

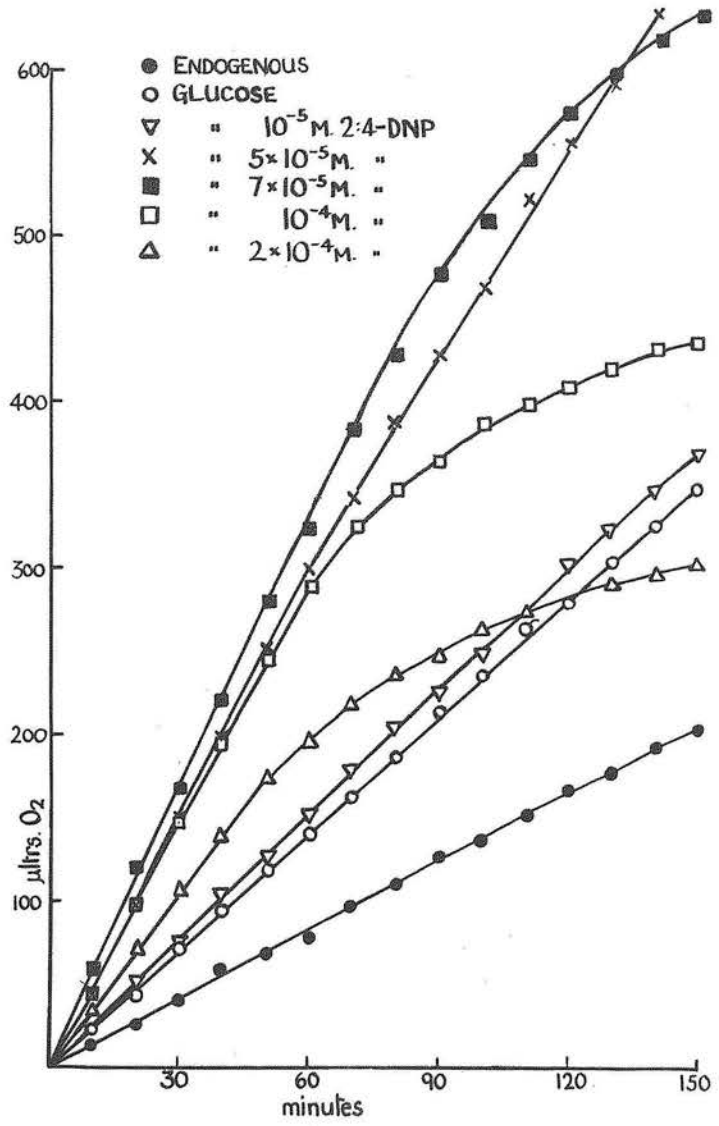
The actions of sodium salicylate on respiration and glycolysis were examined at concentrations varying from 1.0 to 10.0mM. The respiration rates induced at these concentrations were constant for up to 2½ hours, and are expressed as $Q0_2$ values in Table 23. Glycolytic rates are expresssed

in this table as u moles lactic acid produced per mg. protein during the hour over which the experiments were conducted. 1.0mM salicylate was quite ineffective in altering respiratory or glycolytic metabolism. At concentrations between 2 and 4mM definite increases in both processes were observed. These were small in comparison to the effects of 2:4-DNP. The greatest stimulation of respiration occurred at 3mM which induced an average increase of only 37%. The maximum rate of lactic acid production occurred at about the same concentration, and was never greater than twice the control rate. The control of pH was therefore no problem in these experiments and experiment 2 of table 23 shows that this did not fall below 7.2. The net production of α-keto acids into the medium was stimulated by salicylate and increases of up to 150% were induced by 3 - 4mM uncoupler. 10mM salicylate depressed respiration to rates which were rather lower than those of the controls, and over the hour the lactic acid produced into the medium did not differ significantly from that produced by the controls. Graph 21 shows the typical effects of salicylate on endogenous respiration. Both 2mM and 4mM concentrations induced a small initial stimulation followed by inhibition. 10mM salicylate produced marked and immediate inhibition. The effects of salicylate on endogenous respiration are therefore very similar to those observed for 2:4-DNP.



GRAPH 17.

EFFECTS OF 2:4-DNP ON ENDOGENOUS RESPIRATION (7 mg. CELL PROTEIN)



GRAPH 16.

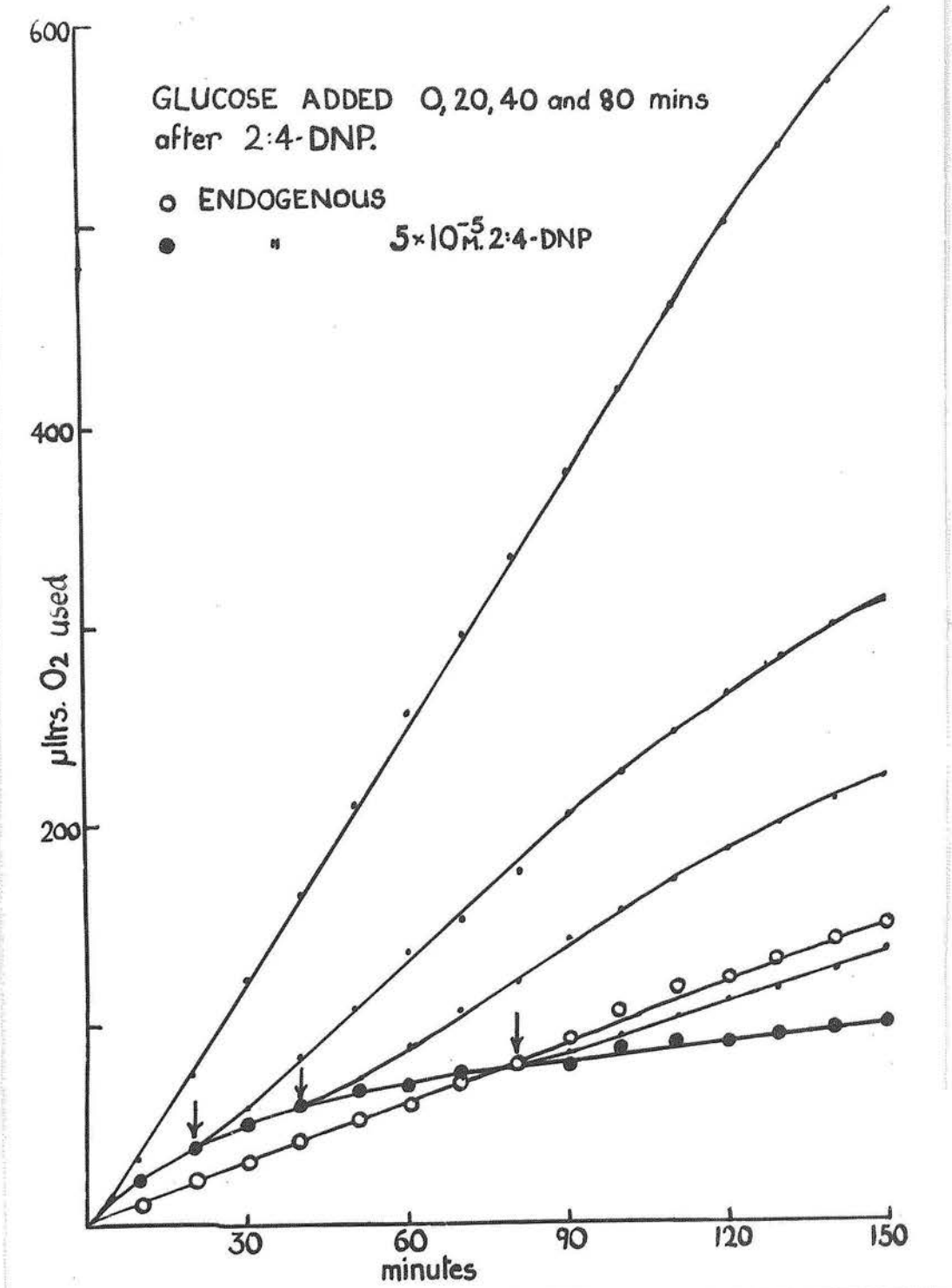
EFFECTS OF 2:4-DNP ON OXIDATION OF GLUCOSE (12 mM) BY 7.0 mg. CELL PROTEIN.

TABLE 19

EFFECTS OF 2:4-DNP ON GLUCOSE METABOLISM UNDER MANOMETRIC CONDITIONS

2:4-DINITROPHENOL	-	0.01	0.05	0.07	0.10	0.20mM
Exogenous respiration	348	368	663	620	437	303
Endogenous respiration	181	193	134	117	117	77
Glucose oxidised	1.25	1.31	3.94	3.74	2.39	1.68
Lactic acid produced	3.65	6.00	19.32	24.21	24.54	24.07
α-keto acids produced	0.15	0.09	0.28	0.27	0.21	0.27
Glucose accounted for	3.15	4.35	13.74	15.98	14.76	13.85
Glucose used	4.92	6.24	15.04	17.30	14.92	14.58
Glucose unaccounted for	1.77	1.89	1.30	1.32	0.16	0.73
% of glucose used which is unaccounted for	36	30	8	7	1	5
Final pH of media	7.2	7.1	6.7	6.4	6.45	6.4
% staining						
With glucose	7.7	7.3	9.0	13.5	13.3	15.3
Without glucose	6.3	7.3	11.0	10.5	16.0	15.5
% showing reductive ability with tetrazolium under nitrogen						
With glucose	89.0	-	-	-	-	81.5
Without glucose	95.7	-	-	-	-	78.2

7.0 mg. protein per flask.



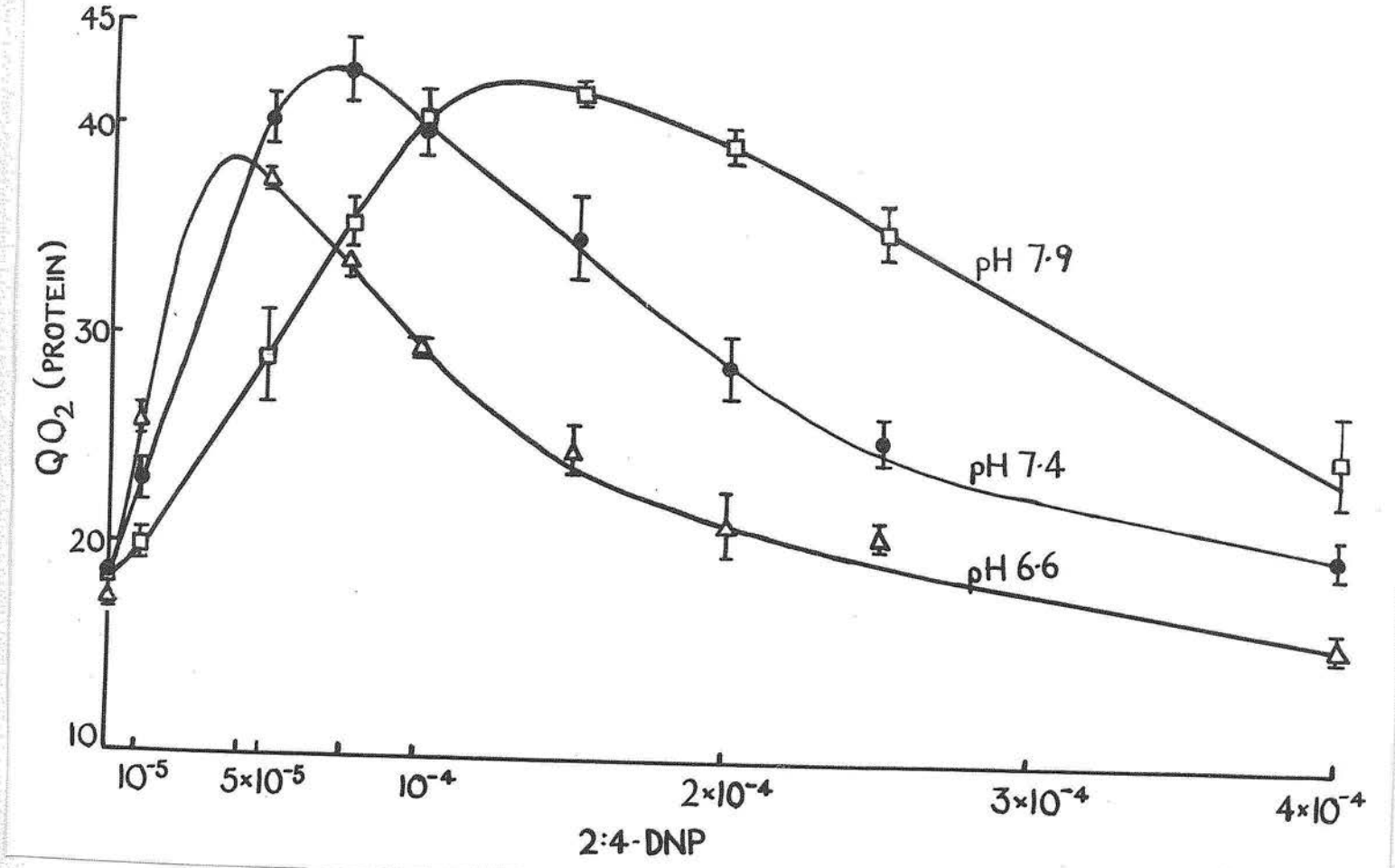
GRAPH 18.

EFFECTS OF ADDITION OF GLUCOSE (10mM) AFTER 2:4-DNP. 6.0 mg. CELL PROTEIN PRESENT.

TABLE 20

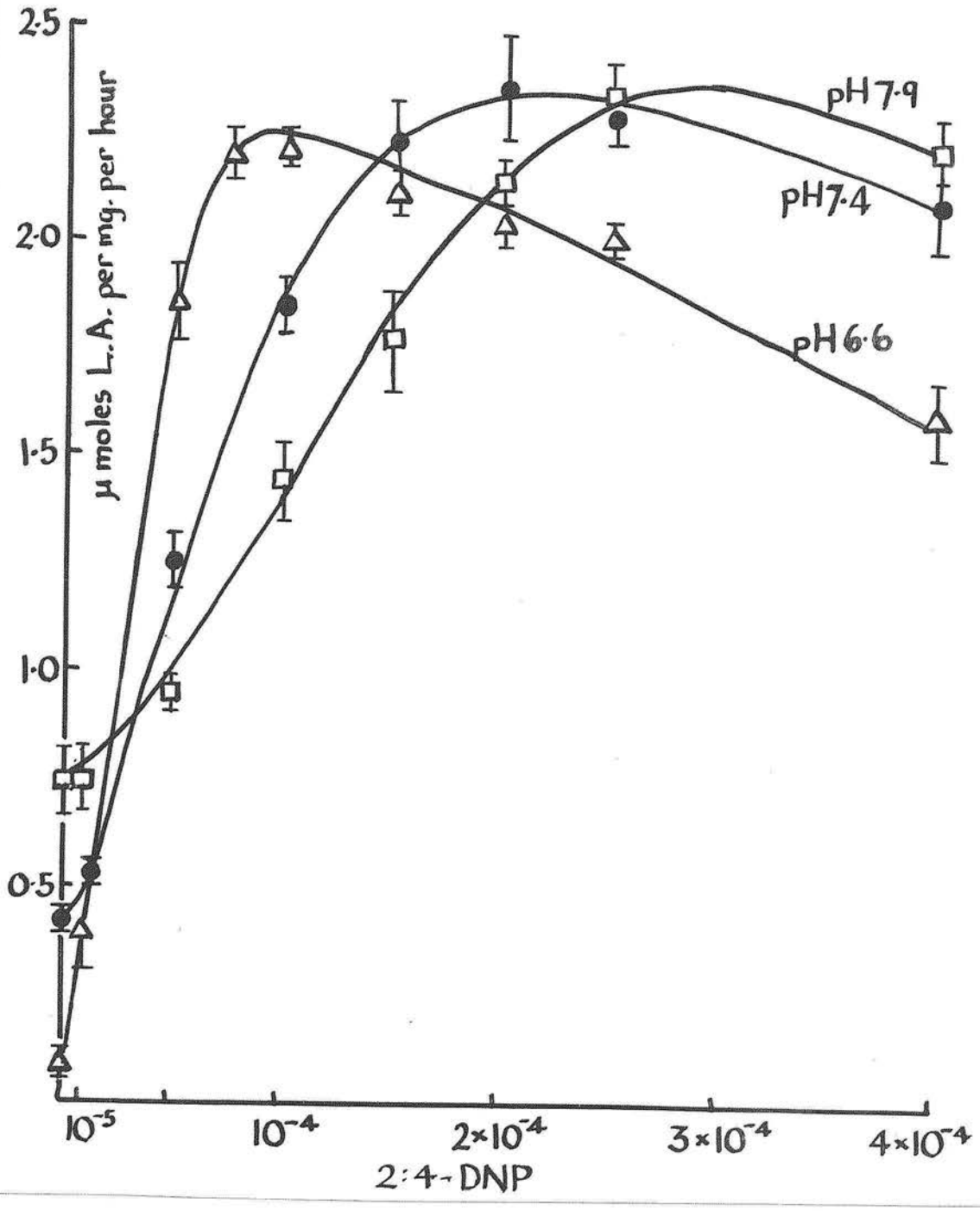
EFFECTS OF ADDITION OF GLUCOSE AT VARIOUS INTERVALS AFTER ADDITION OF 0.05mM 2:4-DNP.

	Time (mins.)	$Q0_2$ for 2:4-DNP induced endogenous respiration	% staining after 150 mins.	$Q0_2$ on adding glucose	% staining after 150 mins.
EXPERIMENT 1	0	22		38.5	7.2
6.0 mg. protein per flask	20	10		23	12.0
	40	7		15	15.3
	80	3.3	14.4	8.7	14.7
EXPERIMENT 2	0	25		37	9.4
7.2 mg. protein per flask	15	14		30	11.7
	30	9		18	10.5
	60	5	11.0	11	10.0



GRAPH 19.

THE EFFECTS OF pH ON THE ACTION OF 2:4-DNP ON RESPIRATION RATE.



GRAPH 20

THE EFFECTS OF pH ON THE ACTION OF 2:4-DNP ON THE RATE OF PRODUCTION OF LACTIC ACID.

TABLE 21

PASTEUR EFFECT, AND EFFECT OF 2:4-DNP ON ANAEROBIC GLYCOLYSIS.

	Q_{O_2} Aerobic L.A.		Q_{N_2} Anaerobic L.A.				A	B	C
2:4-DNP	-	0.2mM	-	0.1	0.15	0.2mM			
7.3mg. Prot. 30 mins.	0.32	2.14	1.20	1.72	1.62	1.62	3.8	1.32	1.36
6.6 mg. Prot. 30 mins.	0.47	2.43	1.60	1.76	1.85	1.82	3.4	1.32	1.16
6.7 mg. Prot. 45 mins.	0.46	2.10	1.41	1.71	1.98	1.35	3.1	1.06	1.42
9.5 mg. Prot. 20 mins.	0.38	2.52	1.47	1.86	1.92	1.68	3.9	1.32	1.30
8.6 mg. Prot. 30 mins.	-	-	1.38	1.02	1.36	0.99	-	-	1.0
9.5 mg. Prot. 20 mins.	0.45	2.31	1.08	1.62	1.65	1.32	2.4	1.40	1.52
MEAN	0.42	2.30	1.36	1.61	1.73	1.46	3.3 s.d. 0.5	1.28 s.d. 0.11	1.30 s.d. 0.18

Column A indicates the ratio of the rate of anaerobic glycolysis to that of aerobic glycolysis.

Column B indicates the ratio of the rate of 2:4-DNP stimulated aerobic glycolysis to that of 0.15mM 2:4-DNP stimulated anaerobic glycolysis.

Column C indicates the ratio of the rate of 0.15mM 2:4-DNP stimulated anaerobic glycolysis to that of anaerobic glycolysis.

TABLE 22

EFFECTS OF 2:4-DNP ON ANAEROBIC ENDOGENOUS GLYCOLYSIS

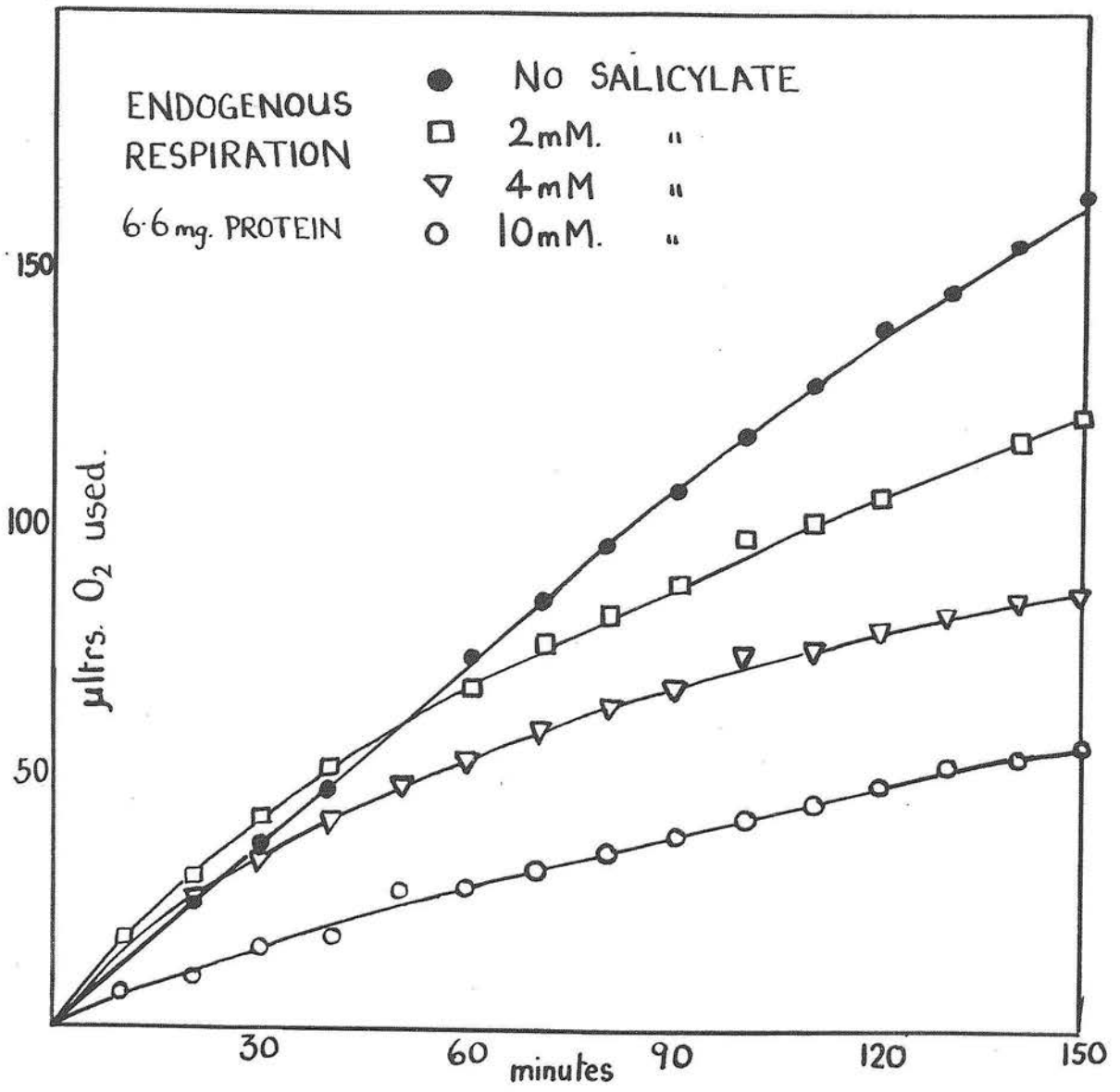
Exp. 1 8.73 mg. protein per flask				
2:4-DINITROPHENOL	-	0.01	0.05	0.10mM
L.A. produced during gassing	1.50			
L.A. after 1 hour	2.75	2.52	2.20	1.88
L.A. produced in 1 hour	1.25	1.02	0.70	0.38 u moles
Aerobic control without 2:4-DNP used 132 u ltrs. O_2 in 1 hour.				
Exp. 2 9.42 mg. protein per flask				
2:4-DINITROPHENOL	-	0.01	0.05	0.10mM
L.A. produced during gassing	1.36			
L.A. after 1 hour	2.87	2.30	2.03	1.70
L.A. produced in 1 hour	1.51	0.94	0.67	0.34 u moles
Aerobic control without 2:4-DNP used 140 u ltrs. O_2 in 1 hour.				

Exp. 3 7.55 mg. protein per flask	Flask 1	Flask 2	Mean
L.A. produced during gassing	1.35	1.15	1.20 u moles
L.A. produced after 1 hour	2.47	2.49	2.48 "
L.A. produced after 1½ hours	2.70	2.83	2.76 "
L.A. produced after 2 hours	2.77	2.62	2.70 "

TABLE 23.

EFFECTS OF SODIUM SALICYLATE ON METABOLISM UNDER MANOMETRIC CONDITIONS

Sodium salicylate	0.0	1mM	2mM	3mM	4mM	10mM	
Exp. 1 6.9 mg. prot.							
$Q0_2$ (exogenous)	19.1	18.2	23.6	26.6	25.4	18.0	u ltrs.
Q lactic Acid	0.33	0.40	0.56	-	0.61	0.42	u moles
Q α-keto Acids	0.03	0.01	0.07	-	0.09	0.04	u moles
Exp. 2 7.1 mg. prot.							
$Q0_2$ (exogenous)	20.8	22.8	21.2	27.3	26.0	-	u ltrs.
Q lactic Acid	0.42	0.42	0.60	0.71	0.77	-	u moles
pH after experiments	7.3	7.3	7.3	7.2	7.2	-	
Exp. 3 6.6 mg. prot.							
$Q0_2$ (exogenous)	18.9	20.0	24.5	26.8	-	15.0	u ltrs.
Q lactic Acid	0.40	0.37	0.52	0.80	-	0.42	u moles
Q α-keto Acids	0.04	0.06	0.10	0.11	-	0.08	u moles



GRAPH 21.

THE EFFECTS OF SODIUM SALICYLATE ON ENDOGENOUS RESPIRATION.

C. EFFECTS OF 2:4-DNP ON 14-C GLUCOSE INCORPORATION AND 14-C AIB UPTAKE

(i) Incorporation of 14-C glucose under aerobic conditions

Table 24 shows that under aerobic conditions, the cells readily incorporated 14-C glucose into acid-precipitable material at a constant rate of 6.9 - 7.9 ug. per mg. cell protein hour for 1½ hours at least. The nature of the cellular components into which the 14-C was incorporated was not determined. The constancy of the rate, however, is intriguing and suggests that whatever the precursors into which the 14-C passes may be, they cannot be diluted by substantial quantities already present in the cell. Furthermore, it indicates that endogenous components for synthesis are present in sufficient quantity to support synthetic reactions such as these into which the 14-C passes, for some time. That these are synthetic reactions rather than mere turnover of cellular material is shown by data also in Table 24 from experiments in which 14-C labelled cells were washed quickly and incubated for a further period in medium containing "cold" glucose. In no case was the amount of 14-C in the acid-precipitable cell material substantially reduced by this treatment. This indicates that turnover of this material is minimal.

Graph 22 shows the results of 2 experiments on the effects of 2:4-DNP on 14-C glucose incorporation. Each point on the Graph was derived from one flask only. The uncoupler markedly inhibited incorporation, with the establishment of new and constant rates. The average inhibitions imposed by 0.05, 0.10 and 0.20mM were 23%, 71% and 77% respectively. Further experiments undertaken to relate 14-C glucose incorporation to respiration and glycolysis are presented in Table 25. These were conducted over 1 hour. In the first two experiments the cells were incubated in the usual phosphate buffered Krebs Ringer solution, and it can be seen that over 1 hour, 2:4-DNP induced significant inhibition of incorporation at all concentrations tested.

At 0.05mM, a 25 - 32% inhibition was found. At 0.10mM the inhibition was 65 - 75%. However, even at much higher concentrations incorporation was not completely suppressed. At 0.5mM for instance, the inhibition only increased to 83%.

Experiment 3 of Table 25 shows results from an experiment in which the cells were suspended in a phosphate buffered complete nutrient solution to which 14-C glucose was added. The results do not permit firm conclusions to be drawn on differences between the two media, but the respiration rates of the controls ($Q0_2$:23) were higher than those observed in Krebs Ringer solution ($Q0_2$:18), although the maximum 2:4-DNP stimulated rates were similar. Incorporation of 14-C in the controls without 2:4-DNP was also substantially greater than in cells in Krebs Ringer Phosphate and averaged 10.8 ug. glucose per mg. cell protein hour. This may indicate that overall synthesis is proceeding faster in the nutrient medium, but further experiments are required to confirm this. The effects of 2:4-DNP were substantially similar to those on cells in Krebs Ringer Phosphate, about 70% inhibition occurring at 0.10mM 2:4-DNP, with little further inhibition at higher concentrations.

(ii) Incorporation of 14-C glucose under anaerobic conditions

As shown in Table 26, 14-C glucose added to cells under anaerobic conditions was incorporated into acid precipitable material. In the two experiments conducted, glucose incorporation was about 2.5 ug. per mg. cell protein hour and therefore considerably less than that earlier observed under aerobic conditions (page 114). The results, however, cannot be directly compared, because of obvious differences in metabolic and synthetic pathways for glucose under the two different conditions. Despite its small and variable effect on anaerobic glycolysis, 2:4-DNP produced a very marked inhibition of 14-C incorporation at 0.10 and 0.20mM concentrations. In the first case, the average inhibition was 34% and in the second 44%.

(iii) Uptake of 14-C α-aminoisobutyric acid

The non-metabolisable amino acid, α-aminoisobutyric acid (AIB) has been widely used in studies of amino acid uptake. Graph 23 shows that the cells rapidly removed 14-C AIB from glucose-containing medium and accumulated it to an intracellular concentration of 50mM or more. In all experiments, the initial AIB concentration in the medium was 5.5mM and the final concentration never less than about 5.1mM. The rate of uptake progressively decreased with time and was not constant over any period which could be conveniently measured. The rate of uptake varied with temperature. At 4°C no accumulation occurred and the intracellular 14-C AIB concentration was equal to the concentration in the medium. This equilibration occurred rapidly within five minutes or less and no further uptake was observed over 45 minutes and an hour. At 25°C uptake was 35 - 40% slower than the initial rate at 37°C, but attained the same intracellular concentration within an hour.

Graphs 24 and 25 show that 2:4-DNP was effective in inhibiting uptake. In such experiments and in those under anaerobic conditions, glucose with or without 2:4-DNP was always added 15 minutes before the 14-C AIB was tipped. To obtain intracellular 14-C AIB concentrations which reflected initial rates of uptake as closely as possible, experiments were terminated after ten minutes in most cases. 0.01mM 2:4-DNP was quite ineffective in inhibiting uptake under those conditions. In a few experiments such as that illustrated in Graph 24 small stimulations were observed, but these were not significant. Inhibition of 14-C AIB uptake by 2:4-DNP, reflected that of 14-C glucose incorporation in that 0.10mM 2:4-DNP produced a marked inhibition which was not greatly increased by much higher concentrations of the uncoupler. Graph 25 shows that 0.10mM 2:4-DNP inhibited uptake over ten minutes by about 36% and 0.50mM by 45%. Anaerobiosis inhibited uptake by an average of 44%.

As with 14-C glucose incorporation 2:4-DNP produced a definite inhibitory effect on 14-C AIB uptake under anoxia. This was particularly apparent at 0.1mM and 0.2mM concentrations when the uptakes were rather lower than at similar 2:4-DNP concentrations under aerobic conditions. Table 27 shows that considerable AIB uptake occurred in 10 minutes under anaerobic conditions without exogenous substrate. This amounted to about 40% of the uptake under optimal conditions in which glucose was respired. To test whether substrate level phosphorylations of endogenous substrate were important in this respect, 1.0mM Arsenite which is known to uncouple such phosphorylations (Azzone and Ernster, 1961), was added ten minutes before addition of AIB. No substantial reduction in uptake was observed. 1.0mM Iodoacetate was also ineffective in this respect. It is concluded therefore that 40% of the optimum net AIB uptake in 10 minutes can occur under conditions in which the production of ATP is effectively blocked.

The possibilities of exchange diffusion were not thoroughly investigated. Table 28 is, however, of some interest in this respect, and contains data from an experiment in which cells preloaded with 14-C AIB under optimum conditions were washed and reincubated in glucose containing medium with 5mM glycine. A clear indication was obtained that glycine accelerates the loss of AIB from the cells.

(iv) Effects of sodium salicylate on 14-C glucose incorporation

Table 28b shows the results of two experiments on the effects of sodium salicylate on 14-C glucose incorporation. It is clear that over the concentration range 1.0 to 10.0mM, salicylate is increasingly inhibitory to synthesis, although respiration is not substantially depressed even at 10mM. Incorporation was virtually completely inhibited by 7.0mM salicylate.

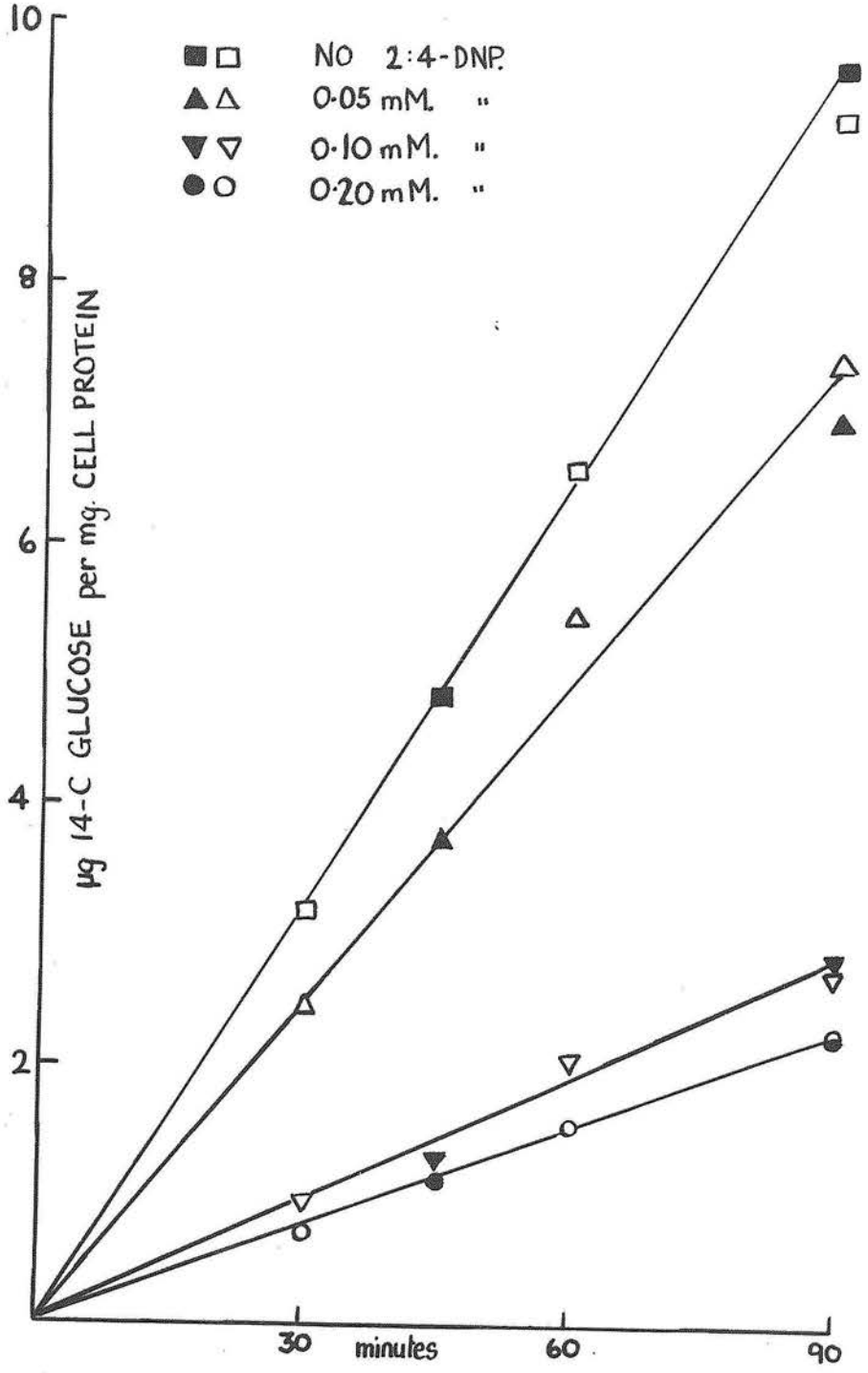
TABLE 24

INCORPORATION OF 14-C GLUCOSE INTO ACID-PRECIPITABLE CELL MATERIAL.

Time	30 mins.		60 mins.		90 mins.	
Exogenous respiration	76	70	138	137	210	205 μl to O_2 used.
Total 14-C activity in acid-ppt. material (counts per 100 secs.)	910	842	2098	1948	2778	2940
ug. 14-C glucose incorporated	27	25	62	58	82	87
Rate of incorporation (ug. glucose/mg. protein hr.)	6.9	6.4	7.9	7.4	7.1	7.5
7.8 mg. protein per flask						

TURNOVER OF 14-C GLUCOSE IN ACID PPT. CELL MATERIAL

	1	2	3
ug. 14-C glucose present in 7.5 mg. cell protein after 30 minutes incubation and washing	33	29	31
ug. 14-C glucose present after a further 30 minutes incubation in "cold" glucose-containing medium	28	30	35



GRAPH 22.

EFFECTS OF 2:4-DNP ON 14-C GLUCOSE INCORPORATION INTO ACID-PRECIPITABLE MATERIAL.

TABLE 25

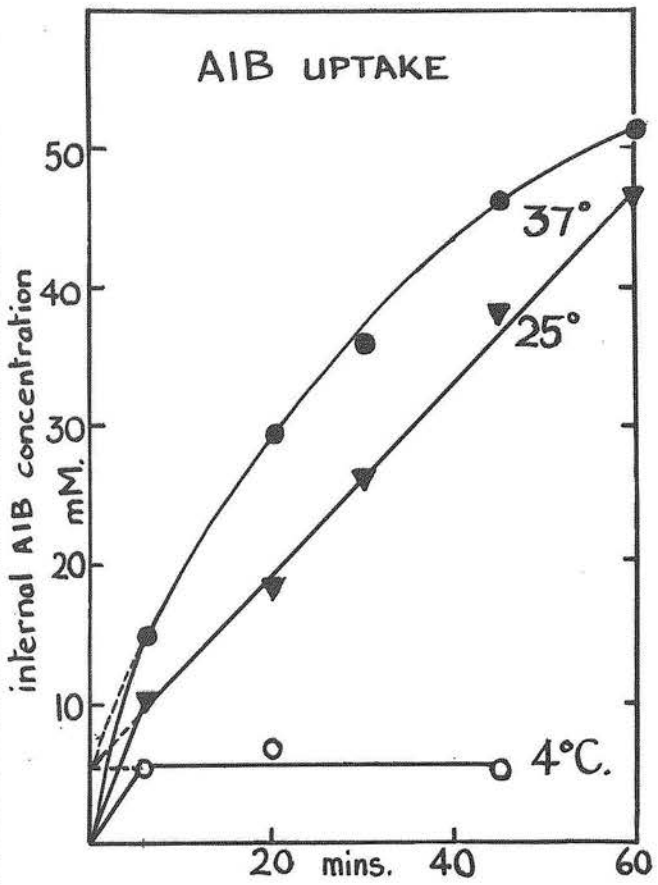
EFFECTS OF 2:4-DNP ON 14-C GLUCOSE INCORPORATION

2:4-DINITROPHENOL	-	0.05	0.10	0.20	0.50mM
Exp. 1 9.6 mg. 1 hour					
Exogenous respiration	172	374	383	298	173
Lactic Acid production	3.65	13.20	17.52	20.13	17.82
14-C activity in acid-ppt. material c.p. 100 secs.	1810	1352	633	462	318
ug. 14-C glucose incorp.	63	47	22	16	11
Rate of incorporation ug. incorp./mg.prot.hour	6.6	4.9	2.3	1.6	1.1
Percentage inhibition	0	25	65	75	83
Exp. 2 6.2 mg. 1 hour					
Exogenous respiration	117	251	234	196	113
Lactic Acid production	2.62	7.80	11.72	13.65	10.19
14-C activity in acid-ppt. material c.p. 100secs.	1409	932	372	258	239
ug. 14-C glucose incorp.	54	36	14	10	9
Rate of incorporation ug. incorp./mg.prot.hour	8.7	5.8	2.3	1.6	1.5
Percentage inhibition	0	32	74	81	83
Exp. 3 8.2 mg. 1 hour					
Exogenous respiration	191	340	323	-	173
14-C activity in acid-ppt. material c.p. 100secs.	2453	1701	563	-	392
ug. 14-C glucose incorp.	88	61	20	-	14
Rate of incorporation ug. incorp./mg.prot.hour	10.8	7.4	2.4	-	1.7
Percentage inhibition	0	32	77	-	84

TABLE 26

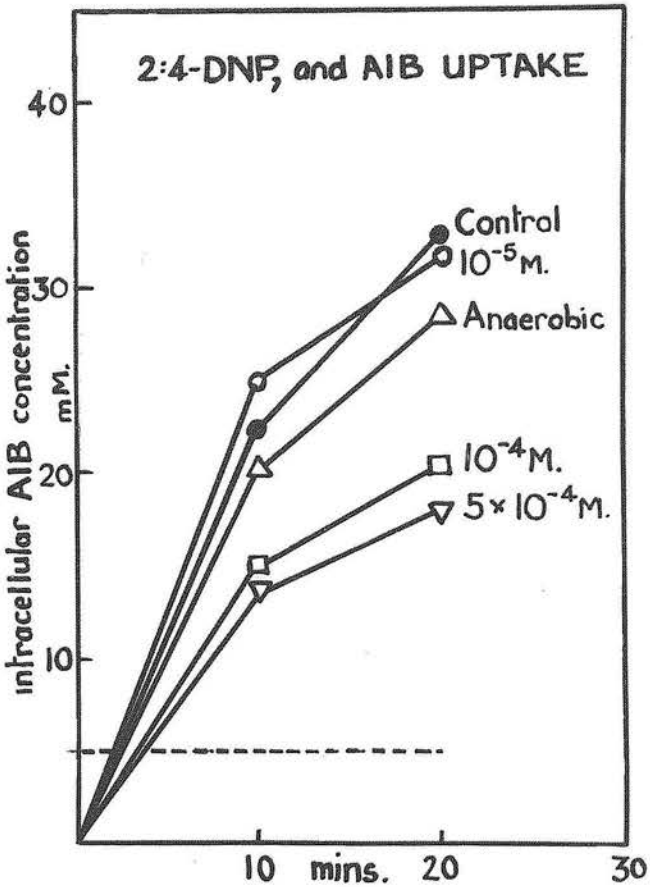
INCORPORATION OF 14-C GLUCOSE INTO ACID-PPT MATERIAL IN 1 HOUR UNDER ANAEROBIC CONDITIONS

2:4-DNP	-----		0.05mM	0.10mM		0.20mM	
EXPERIMENT 1 7.03mg. PROTEIN							
counts/1000 secs.	5552	5438		3842	3608	3147	2342
u.g. 14-C glucose incorp.	18.5	18.1		12.8	12.1	10.5	7.8
u.g. 14-C glucose /m.g. protein	2.64	2.58		1.82	1.72	1.50	1.11
EXPERIMENT 2 6.32mg. PROTEIN							
counts/1000 secs.	4175	4706	4053	2950	2813	2572	2899
u.g. 14-C glucose incorp.	13.9	15.7	13.5	9.8	9.4	8.6	9.7
u.g. 14-C glucose /m.g. protein	2.21	2.50	2.14	1.58	1.50	1.36	1.54
Σ 14-C glucose /m.g. protein	2.48		2.14	1.65		1.38	



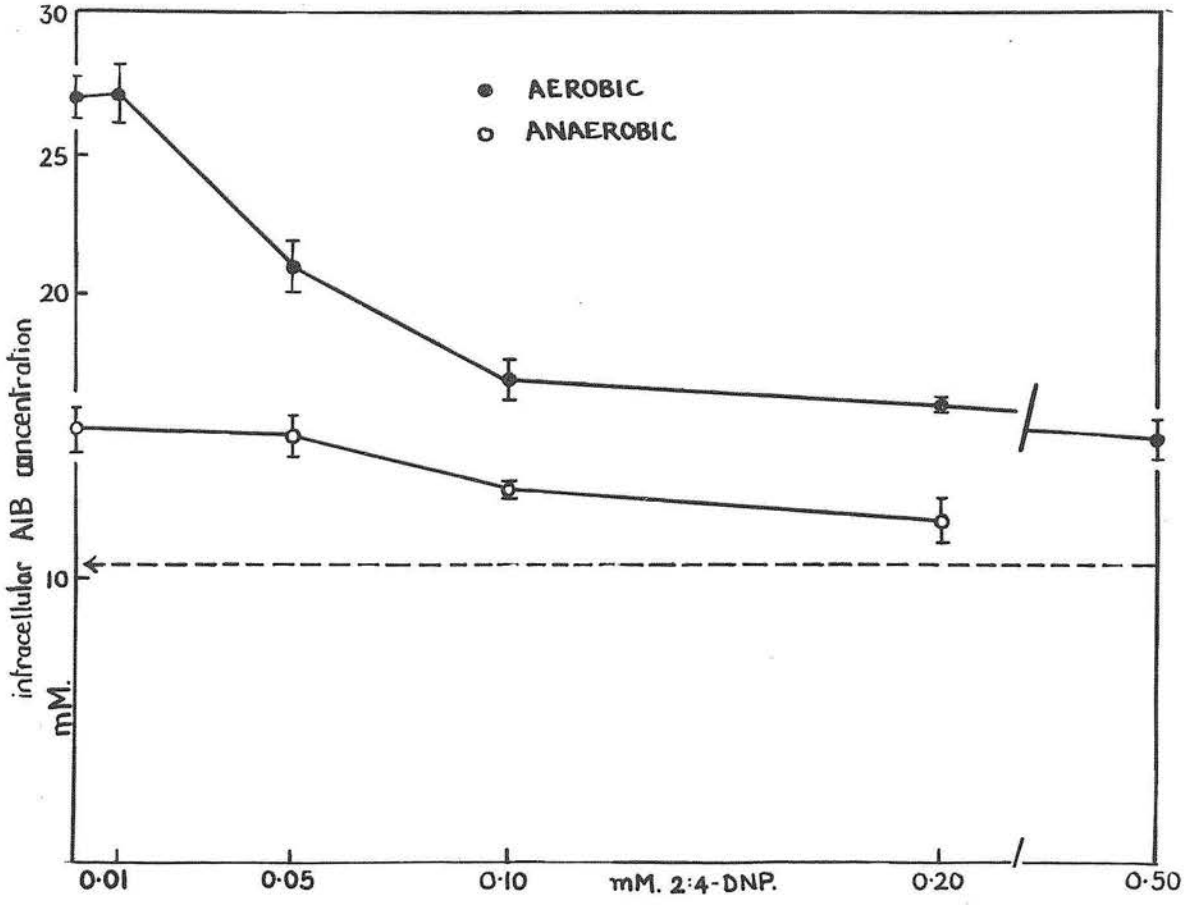
GRAPH 23

THE UPTAKE OF 14-C AIB



GRAPH 24

EFFECTS OF 2:4-DNP ON THE UPTAKE OF 14-C AIB.



GRAPH 25

DATA FROM 4 EXPERIMENTS ON THE EFFECTS OF 2:4-DNP ON AIB UPTAKE.

Standard Deviations are shown. The broken line marks uptake under maximum inhibition of ATP production.

TABLE 27

14-C AIB UPTAKE IN 10 MINUTES UNDER MAXIMAL ENERGY INHIBITION

Gas Phase	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Aerobic	
Substrate	Endog.	Endog.	Endog.	Glucose	Glucose	
Anti Metabolite	-	Arsenate (1mM)	IOD (1mM)	-	-	
Exp. 1	11.4	10.6	-	15.4	28.0	mM
	-	9.8	-	-	27.0	mM
Percentage of aerobic uptake	41%	37%	-	56%	100%	
Exp. 2	10.6	11.5	11.0	-	25.6	mM
	10.0	-	8.9	-	27.0	mM
Percentage of aerobic uptake	39%	43%	38%	-	100%	
Exp. 3	-	11.6	11.8	-	29.8	mM
Percentage of aerobic uptake	-	39%	40%	-	100%	

TABLE 28

EFFECT OF EXTRACELLULAR GLYCINE ON LOSS OF INTRACELLULAR 14-C AIB

	Intracellular 14-C AIB (mM)	
	No extracellular GLYCINE	5mM GLYCINE
0 minutes	48	48
5 minutes	40	38
10 minutes	44	30
20 minutes	44	24

TABLE 28b

EFFECTS OF SODIUM SALICYLATE ON 14-C GLUCOSE INCORPORATION OVER 1 HOUR.

Sodium salicylate	-	1mM	2mM	4mM	7mM	10mM
Exp. 1 7.3 mg. prot						
Exogenous respiration ($\mu l\ O_2$)	162	158	190	205	179	133
ug. 14-C glucose incorp.	53.2	51.2	38.9	23.1	3.6	2.1
Rate of incorporation (ug. 14-C glucose/mg.hr.)	7.3	7.0	5.3	3.2	0.5	0.3
Percentage inhibition		4%	27%	56%	94%	96%
Exp. 2 7.0 mg. prot						
Exogenous respiration ($\mu l\ O_2$)	136	149	163	188	140	110
ug. 14-C glucose incorp.	47.1	49.3	29.8	28.3	4.8	1.5
Rate of incorporation (ug. 14-C glucose/mg.hr.)	6.7	7.0	4.2	4.1	0.7	0.2
Percentage inhibition			36%	39%	90%	97%

D. EFFECTS OF 2:4-DNP ON INTRACELLULAR ADENINE NUCLEOTIDES

The manipulations required for extraction and estimation of the nucleotides made it difficult to handle more than 6 cultures at once. As a result, information has been obtained from several experiments in which only single flasks were estimated for each concentration of uncoupler. As stated earlier, all experiments in which significant breakdown of marker nucleotides occurred during extraction, were discarded. Results of 3 experiments are presented in Table 30A. These show that 2:4-DNP produced significant changes in the proportions of the nucleotides. In the absence of 2:4-DNP, cells metabolising glucose had an ATP:ADP ratio of approximately 90:10 and only trace quantities of AMP were present. Addition of 0.05mM uncoupler resulted in a small decrease in ATP and increases in ADP and AMP, giving an average ATP:ADP:AMP ratio of 75:20:5. This trend increased at higher 2:4-DNP concentrations, and 0.1mM and 0.2mM induced ratios of 66:26:8 and 56:32:12 respectively. A satisfying aspect of the results is the relative constancy of the total adenine nucleotide content at about 0.03u moles per mg. protein.

It was of importance to determine whether the patterns noted after 30 minutes exposure to the uncoupler were steady state patterns or merely transitional. Four experiments, each with a miscellany of 2:4-DNP concentrations were undertaken, sacrificing cultures at 15 and 60 minutes after addition of uncoupler. The results (Table 30B) show that 2:4-DNP changes the nucleotide proportions within 15 minutes (and probably much less) and that further change does not occur over a period of one hour. Further experiments have in fact shown that this is entirely dependent upon the pH maintaining itself around the physiological level.

TABLE 30

EFFECTS OF 2:4-DNP ON ADENINE NUCLEOTIDES

A. NUCLEOTIDES 30 MINUTES AFTER ADDITION OF 2:4-DNP.

	u moles per 10 mg. protein							
	2:4-DNP	ATP	ADP	AMP	TOTAL	Ratio: ATP :	ADP :	AMP
Exp. 1	-	.278	.028	.003	.309	90	9	1
	0.05mM	.220	.048	.014	.282	78	17	5
	0.10	.212	.077	.026	.315	67	24	8
	0.20	.162	.089	.019	.270	60	33	7
	0.20	.163	.105	.044	.312	53	34	13
Exp. 2	-	.285	.033	-	.318	90	10	-
	0.05	.220	.059	.019	.298	74	20	6
	0.10	.195	.073	.024	.292	67	25	8
Exp. 3	-	.266	.032	.002	.300	88	11	1
	-	.263	.027	.002	.292	90	10	-
	0.05	.193	.062	.013	.268	72	23	5
	0.10	.180	.081	.020	.281	63	29	7
	0.20	.157	.080	.043	.280	56	29	15
Means								
	-	.273	.030	.002	.305	90	10	-
	s.d.	.009	.003	.002				
	0.05	.211	.056	.015	.282	75	20	5
	s.d.	.013	.006	.003				
	0.10	.196	.077	.023	.296	66	26	8
	s.d.	.013	.003	.003				
	0.20	.161	.092	.035	.288	56	32	12
	s.d.	.003	.010	.011				

Continued

TABLE 30 (continued)

B. EFFECTS OVER ONE HOUR

Data from 4 experiments
(see page 118)

2:4-DNP	Time (minutes)		u moles per 10 mg. protein ATP	ADP	AMP	Flasks
-	15		.260	.027	-	2
		a.d.	.004	.004	.002	
	60		.277	.031	.003	2
		a.d.	.015	-	.001	
0.05mM	15		.225	.067	.013	3
		s.d.	.010	.006	.004	
	60		.218	.058	.024	3
		s.d.	.015	.004	-	
0.10mM	15		.183	.076	.023	3
		s.d.	.016	.010	.008	
	60		.192	.073	.016	3
		s.d.	.003	.002	.002	
0.20mM	15		.166	.101	.036	3
		s.d.	.005	.009	.006	
	60		.154	.097	.026	3
		s.d.	.003	.008	.003	

E. EFFECTS OF 2:4-DNP ON ATPase ACTIVITY

ATPase activity was measured conventionally by the release of Pi from ATP added to cell homogenates. In the course of such measurements chromatographic separation of the adenine nucleotides showed that considerable quantities of AMP appeared during the reaction. Controls showed that this was a result of enzyme action and was not due to spontaneous breakdown of ADP or ATP. The most obvious interpretation of this was that the appearance of AMP was due to adenylate kinase. This has not however been definitely established. The following information has been established and is of some relevance:- Isolated mitochondria as well as homogenates hydrolyse ATP with the appearance of AMP as well as ADP. When ADP is added, AMP and Pi appear in the medium. On a few occasions a slight trace of ATP has been detected but this is not usually the case. The release of Pi from added ADP is slow compared with its release from ATP, and it is only fractionally stimulated by 2:4-DNP (Table 29). These findings are indicative of adenylate kinase action, AMP and ATP being formed from ADP with subsequent hydrolysis of ATP by ATPase activity to ADP with the release of a Pi. Adenylate kinase activity has been reported for many mitochondrial systems (Kielley and Kielley, 1951; Siekevitz and Potter, 1953). In most reported measurements of ATPase activity the presence of adenylate kinase systems has been ignored. If the production of AMP in the current experiments is due to adenylate kinase activity, then the release of Pi from ATP may be taken as a true index of ATPase activity for Pi is neither gained nor lost during adenylate kinase action. If the enzyme is not adenylate kinase, then the stimulation of ATPase activity by 2:4-DNP reported in the following must be rather less than calculated, as the release of Pi from added ADP is fractionally stimulated by 2:4-DNP (Table 29).

Initial experiments established that the rate of release of Pi was linear with time if the rate did not exceed about 1.25 u moles Pi released in 7 minutes. Details of the controls used in these experiments are recorded on page 66. Table 31 shows the results of five experiments on the effects of 2:4-DNP on the release of phosphate from ATP by cell homogenates. Three experiments involved cell homogenates which had been prepared in distilled water, and two in which the cells had been broken in isotonic sucrose. In all the experiments carried out, 2:4-DNP very markedly increased the release of Pi, the maximum activity being induced by 0.20mM uncoupler. The average maximum increase of activity in homogenates prepared in distilled water was 170%, and that in sucrose was 310%. Table 31 shows that this difference was principally due to differences in the ATPase activities of the controls without 2:4-DNP, and that the maximum 2:4-DNP induced rates were very similar. It is well established that damage to the mitochondrial membranes during preparation stimulates ATPase activity (Charnock and Opit, 1962). It would appear that preparation in distilled water is unsatisfactory in this respect.

TABLE 29

EFFECTS OF 2:4-DNP ON RELEASE OF Pi FROM ADP AND ATP

	u moles Pi released per mg. protein in 7 mins.	
2:4-DNP		0.2mM
ADDED SUBSTRATE		
ADP	0.020 0.018	0.033 0.027
ATP	0.053 0.062	0.212 0.182
ADP	0.011 0.017	0.018 0.040
ATP	0.067 0.065	0.243
½ ADP ½ ATP	0.016 0.062	0.029 0.212

TABLE 31

EFFECTS OF 2:4-DNP ON ATPASE ACTIVITY IN HOMOGENATES

2:4-DINITROPHENOL	u moles Pi released in 7 minutes					0.2mM 2:4-DNP induced activity / control activity
	---	0.05	0.10	0.20	0.50mM	
5.0 mg. protein. Cells broken in distilled water	0.360	0.542	0.821	0.892	0.615	2.45
4.6 mg. protein. Cells broken in distilled water	0.365	-	0.783	0.978	0.545	2.7
3.2 mg. protein. Cells broken in distilled water	0.263	-	0.620	0.753	0.543	2.9
4.7 mg. protein. Cells broken in sucrose	0.23	-	0.85	1.05	0.79	4.5
4.3 mg. protein. Cells broken in sucrose	0.24 0.28	- -	0.97 0.82	0.97 1.08	0.80 0.78	3.9

Cells broken in	Distilled H_2O		Sucrose	
2:4-DINITROPHENOL	---	0.2mM	---	0.2mM
Pi released per mg./min.	0.010	0.026	0.007	0.032
	0.011	0.030	0.008	0.032
	0.012	0.033	0.009	0.036

D I S C U S S I O N

The foregoing experiments have shown that the uncouplers of oxidative phosphorylation, 2:4-dinitrophenol and sodium salicylate, induce significant changes in a variety of metabolic activities - respiration, glycolysis, transport, synthesis, growth and the enzymic and adenine nucleotide constitution of L cells.

Two different experimental systems have been used. A growth system with cells growing as stationary monolayers in bicarbonate-buffered nutrient medium equilibrated with 5% carbon dioxide in air; and a considerably more artificial system in which cells have been subjected to manometric conditions as single cell suspensions in phosphate buffered balanced salt solution equilibrated with air. Despite these divergences the findings from both systems are in general agreement. As more information has been obtained from the manometric system, this is discussed first. Where applicable, the general principles established here are applied to interpretation of the growth experiments in the second section of the discussion.

DISCUSSION

MANOMETRIC EXPERIMENTS

(i) REGULATORY MECHANISMS FOR GLUCOSE METABOLISM

The pattern of glucose metabolism under manometric conditions is in general similar to that reported for this cell type by other authors. Calculated on a dry weight basis for convenient comparison, the respiration rate was 13.5 s.d. 0.7. As these results were obtained over a period of two years, they establish the metabolic stability of the cell line to the growth conditions used. In this respect Danes and Paul (1961) have reported that Danes also obtained consistent results with cells from a semi-continuous suspension culture. A further factor in the attainment of such consistently reproducible respiration rates is that the omission of serum eliminates a major source of variance in the growth media. Phillips and Andrews (1960) in fact demonstrated that supplementation of growth media by different batches of serum could impose marked changes in metabolism which were obvious on examining respiration and glycolysis under manometric conditions. The respiration rate (dry weight basis) of L cells varied in such experiments from 1.1 to 8.1 in phosphate buffered salt solution with glucose, values which are considerably lower than those obtained in the present experiments. The literature on the L cell reveals substantial differences in the respiration rates reported by various authors. Whitfield and Rixon (1960) for example obtained values of 7.0 to 9.0 while Phillips and Pace (1954) reported a mean rate of 4.8. Phillips and Feldhaus (1956) noted that 24 hours after renewal of the growth medium (which contained 40% serum and 20% chick embryo extract) the respiration rate measured in Krebs Ringer Phosphate with glucose was 9.3, a value which decreased on subsequent days to 4.3 after 120 hours. They concluded that a general relationship existed between respiration and growth rate. This idea was further developed by Phillips and Andrews (1960) who noted a respiration rate at 37°C of 4.8 for cells which had been growing slowly at 31°C, compared with 7.0 for cells grown at 37°C. In contrast,

Danes and Paul (1961) observed a maximum respiration rate of about 20.5 when cell growth had ceased 120 hours after renewal of nutrient medium, compared with a rate of 3.2 after the first 48 hours when the rate of growth was maximal. (For convenient comparison, these figures for the work of Danes and Paul have been obtained by assuming that the dry weight of 10^6 cells is of the order of 0.45 mg - the average of the data from this laboratory and several others on the dry weight of L cells. The further assumption has been made that the dry weight per cell is the same at 48 hours during the phase of log growth as at 120 hours during the stationary phase. The calculated figures should therefore be taken as approximate.)

Some of the differences in the results presented by Phillips and workers and those of Danes and Paul (1961) may be due to the fact that the latter workers used a Cartesian Diver technique for measuring respiration whereas the former used conventional Warburg manometry. In general, the results of Danes and Paul appear to be better established. In both cases, however, it is clearly shown that respiration rate is readily altered by growth conditions, media and other environmental factors. This, coupled with the observation of Rucker et al (1963) that different authors give different dry weights for L cells, make divergences in the reported respiration rates understandable. A further factor is the possibility of respiratory aberrations in certain lines: Phillips and Terryberry (1958) for example, report rates of 13.6 and 4.7 (live cell dry weight basis) for two lines of L cell examined manometrically in phosphate balanced salt solution (pH 7.4) with 10% serum present.

In the present study the rate of aerobic glycolysis at pH 7.4 (0.28 s.d. 0.04 u moles lactate per mg dry weight hour) is low and much more variable than respiration rate. Although the reported values for the L cell vary considerably - 0.26 (Phillips and Terryberry, 1958), 0.3 - 1.8 (Phillips and Andrews, 1960), 0.13 (Phillips and Feldhaus, 1956) - they establish the general fact that aerobic glycolysis is characteristically low compared with that of

tumour cells (Woods, Sanford, Burk and Earle, 1959).

A feature then of the present study is that under constant and defined growth and manometric conditions at pH 7.4, the aerobic metabolism of glucose by the L cell is predictable within narrow limits. It has been shown however that this metabolic pattern can be readily altered in a distinct and characteristic manner by each of the following:-

(1) Change in pH of the suspension media
(2) Anaerobiosis
(3) Addition of uncouplers under anaerobic conditions
(4) Addition of uncouplers under aerobic conditions.

Before considering these in detail, some current concepts of the regulation of metabolism will be discussed relative to the basic pattern of L cell metabolism at pH 7.4.

As both respiration and aerobic glycolysis can be greatly stimulated by certain of the treatments denoted above, it may be assumed that the enzyme systems catalysing the interaction between glucose and oxygen are not used to full capacity under normal physiological conditions; in other words, the glucose oxidising and pyruvate reduction systems are not rate limiting in themselves. It has already been observed that both respiration and glycolysis are obligatorily coupled with the synthesis of ATP from ADP and Pi; and the marked increase in respiration rate on uncoupling respiratory chain phosphorylations in most tissues provides good evidence that in general the rate of phosphorylations imposes limitations on the respiration rate and plays an important role in the regulation of metabolism. This removes the problem one step to the question of the rate limiting factors for phosphorylation. The most obvious answer is the rate at which ADP and Pi become available at the sites of phosphorylation. The two parts of the phosphate cycle, phosphorylation and dephosphorylation, must clearly be equilibrated against each other in any stable biological system. Failure to maintain this equilibrium would result in lack of ATP if phosphorylation proceeded too slowly as compared with

dephosphorylation and lack of ADP in the case of too rapid phosphorylation. Thus dephosphorylation which depends directly upon the energy requirements of the cell may regulate the rate of phosphorylation. The manner in which this equilibrium is maintained and its relationship to glycolysis, respiration and exergonic processes is far from clear.

It has been consistently demonstrated that addition of ADP and Pi to sub-cellular systems results in increased rates of phosphorylation which are accompanied by increased glycolysis or respiration, or both (Emmelot and Bos, 1959; Chance and Williams, 1956; Slater and Hulsmann, 1959). The production of artefacts is however inseparably attached to procedures involving the disruption of the cell surface. Data from such experiments, although important in establishing that metabolism may be regulated by the phosphate cycle, is difficult to refer to a composite picture of intracellular metabolism. A survey of the literature suggests in fact, that by varying dilutions and the proportions of exogenous adenine nucleotides, it is possible to implicate at will either ATP, ADP or Pi as rate limiting factors. As Racker and Wu (1959) have pointed out, such experiments are important solely insofar as information from a very large number of artefacts may eventually merge into an acceptable synthesis. The latter authors underlined the importance of comparing sub-cellular investigations with intracellular studies; they observed that within limits the rate of glycolysis of Ehrlich tumour cells was directly related to intracellular Pi, and the paradox that the lowest concentration of Pi found in cells stimulated a reconstructed glycolytic system to produce lactate at a disproportionally high rate. This introduces the important subject of compartmentation within the cell, a concept advanced on several occasions to account for the relationship of glycolysis to respiration. Racker and Wu concluded that at all but very high extracellular Pi concentrations, a high proportion of the intracellular Pi was unavailable for glycolysis, as respiratory chain phosphorylations depleted the extramitochondrial cytoplasm of the ion.

Some support for this comes from the work of Lynen et al (1959) who noted that on plasmolysis and fractionation, actively respiring yeast had a considerably higher proportion of Pi in the particulate areas of the cell than "starved" yeast.

Whether Pi is of as general importance in the regulation of metabolism as these considerations indicate is questionable. Ibsen, Coe and McKee (1958) in a detailed investigation of the Crabtree Effect in tumour cells concluded that the rate of entry of Pi into cells was sufficiently rapid to abolish even any local deficiency which might occur. Shacter (1957) is also of this opinion. Hess (1961) measured reactions of respiratory components in intact cells on addition of glucose and concluded on kinetic grounds that Pi was unlikely to be limiting for glycolysis.

Availability of Pi seems unlikely to be a limiting factor for the metabolism of L cells in the present study. Certainly, a comparison of the ratio of adenosine nucleotides to Pi in the L cell and the apparent ratio in the ascites cell used by Racker and Wu (1959) reveals significant differences.

		u moles per 10 mg. cell protein			
	Pi (ext.)	ATP	ADP	AMP	Pi(int.)
Ehrlich ascites cells	5mM	0.36	0.06		0.27
	80mM				0.70
L cells	25mM	0.272	0.030	0.002	1.12

It will be seen that an increase in the extracellular Pi to 80mM increased the internal Pi considerably in the ascites cell. This was accompanied by an increase in the rate of lactate production to what appears from Racker's data to be near maximal. Even allowing for differences in assay, it seems that not only is there a higher absolute concentration of Pi in L cells at 25mM external concentration, but the ratio of total adenosine nucleotides to Pi is much lower than in ascites cells at very high external Pi. Conclusions

from this may be criticised on the grounds that interpretation of Pi determination in terms of actual Pi concentration is of limited value if the ion is compartmented within the cell. Of more significance perhaps is the finding that where external Pi was reduced by 40%, no diminution of respiration or glycolysis of the L cell was noted (page 48). This is contrary to Racker's observations for the ascites cell and may be taken as circumstantial evidence that the availability of Pi is unlikely to limit metabolism in L cells under normal manometric conditions with an external concentration of 25mM Pi.

It has been suggested (Chance and Hess, 1956) that availability of ATP might control the rate of glycolysis at the level of the kinases. The question therefore arises whether the relatively low glycolytic rate in L cells at pH 7.4 is a result of a limiting rate of hexose phosphorylation. The high steady state ratio of ATP:ADP in L cells (Table 30) indicates that this is unlikely if this ratio is present at the site of kinase activity. Recently however evidence has arisen for a compartmentation of ATP within the mitochondria. (Hess (1961) found for example that mitochondria isolated from glucose-treated ascites cells contain about 3 times the ATP of control samples and concluded from this and kinetic studies that ATP produced by oxidative phosphorylation is not fully fed back to the extramitochondrial cytoplasm but retained in the mitochondria. Further support for this idea comes from work by Balazs, Magyar and Richter (1964) on brain mitochondria. These authors showed that ATP formed in brain mitochondria in vitro is not available for hexokinase reactions but can be released under certain conditions as when ADP is present in excess. (It may be noted that brain mitochondria differ from those of most other tissues in that they show hexokinase activity.) Furthermore, evidence was presented for a separate compartmentation of ATP formed by substrate level phosphorylation and that from respiratory chain phosphorylation by the different responses to dinitrophenol. The assumption that ATP is distributed uniformly throughout the cell and therefore wholly

available for kinase action is therefore questionable. Nevertheless it may be argued that lack of ATP for the phosphorylation of glucose is unlikely to be rate limiting for aerobic glycolysis at pH 7.4, on the grounds that uncoupling which reduces substantially the cellular ATP concentration and decreases the ATP:ADP ratio increases glycolysis and therefore the rate of phosphorylation several-fold. The distribution of ATP is likely to be different in coupled and uncoupled cells; for example in the latter case intramitochondrial ATP might form a considerably smaller proportion of the total ATP than in the former. As uncoupling reduces the total intracellular ATP from about 90% to 56% of the total adenosine nucleotide complement the mitochondria in coupled cells would require to contain a very high proportion of ATP to justify the conclusion that extramitochondrial ATP was in fact greater in the uncoupled cell than in the coupled cell. It may be pointed out that there is no good evidence for more than 10% of the total ATP of glucose respiring cells being in the mitochondria (Hess, 1961).

The hypothesis that lack of ATP for glucose phosphorylation limits the rate of aerobic glycolysis at pH 7.4 is rejected as unlikely.

Recent evidence suggests that a high cellular ATP:ADP ratio is more likely to inhibit glycolysis than to stimulate it. This has developed from the concept of phosphofructokinase as the pacemaker for glycolysis. Suppression of glucose uptake in rat diaphragm by the respiration of fatty acids and pyruvate was noted by Neusholme and Randle (1962). Respiration of these substrates inhibited the phosphorylation of fructose-6-phosphate which regulates the level of glucose-6-phosphate. As hexokinase is inhibited non-competitively by its product glucose-6-phosphate with a Ki as low as 0.1mM (Sols and Crane, 1954), the authors argued that the rate of glycolysis was essentially controlled at the phosphofructokinase step. Some aspects of the kinetics of this enzyme have been elucidated by Passoneau and Lowry (1962). These authors found that the activity of purified rabbit muscle enzyme was inhibited by high

concentrations of ATP and stimulated by addition of Pi, ADP or AMP. Stimulation was effected by relatively low concentrations of the latter compounds. For example, where ATP was present at 2.3mM and Pi at 2mM, addition of 0.144mM ADP increased activity six times. The reduction of ATP to 1.8mM and addition of 0.02mM AMP, 0.06mM ADP and a small increase in Pi increased activity thirty-fold. The kinetics are complex but the authors have suggested that the enzyme has two sites for ATP - a primary active site and a secondary inhibitory site, stimulators such as ADP and AMP may compete with ATP for the second site and release inhibition in this way. Mansour and Mansour (1962) found that phosphofructokinase from Fasciola hepatica showed similar characteristics to those for rabbit muscle enzyme. Further evidence that ADP and AMP can activate phosphofructokinase as well as control the dephosphorylation steps of glycolysis has come from work on sub-cellular fractions of ascites tumour cells (Tiedemann and Born (1963).

There is substantial agreement therefore that the rate of glycolysis is very likely determined by the concentrations of the adenine nucleotides and Pi at the site of the phosphofructokinase reaction and can be stimulated if ATP falls or ADP, AMP or Pi rises. It is proposed that this is an important factor in the metabolism of L cells, and that the low rate of aerobic glycolysis is a consequence of inhibition of phosphofructokinase by high ATP and low ADP concentrations. The control of phosphofructokinase is therefore generally related to the ADP available for the dephosphorylation steps of glycolysis.

Racker and Wu (1959) concluded that the relative rates of respiration and glycolysis were controlled by competition for Pi between these systems. Reasons for believing that this is not the case in L cells have already been given and a more acceptable hypothesis seems to be competition for ADP. That this does occur in reconstructed glycolytic systems with added mitochondria is adequately documented. For example, addition of glycolytic

systems reduce the rate of mitochondrial respiration by competing for exogenous ADP (Gatt, Krimsby and Racker, 1956). Chance (1959) brought forward kinetic evidence that the mitochondrial system had very low affinity for Pi and most substrates compared with its affinity for ADP. He concluded that ADP was the most favourable candidate for delicate regulation of metabolic processes. Furthermore, the relatively low affinity for ADP of phosphoglycerate kinase indicated that mitochondrial activity was more likely to control glycolytic activity than the converse. (Hess (1961), investigating the reactions of respiratory components in intact tumour cells on addition of glucose also concluded that ADP was the rate limiting factor for respiration.

These considerations of metabolic control applied to L cells may be generally summarised: The ATP:ADP ratio is poised at the optimum resultant for cellular reactions under the given conditions and is maintained by a balance between phosphorylations and dephosphorylations. Respiration and glycolysis are coupled to and limited by the rate of phosphorylations. The immediate rate controlling factor for phosphorylations is not Pi, or the availability of ATP for hexokinase, but ADP. The relative rates of glycolysis and respiration are reflections of the different affinities of these systems for ADP as a phosphate acceptor. Control of the rate of glycolysis should be considered as not merely through the availability of ADP for the dephosphorylation steps but a feedback activation of phosphofructokinase by this nucleotide. Alteration of the metabolic pattern in various ways will now be discussed.

(ii) THE EFFECTS OF pH: A SPECULATIVE DISCUSSION

In the presence of an adequate glucose supply, pH has been shown to greatly influence aerobic glycolysis. At pH values below 7.0 little or no lactate is produced into the medium, whereas at 7.9 the rate is as high as 0.57 u moles per mg. dry weight hour. This characteristic response of aerobic glycolysis to pH has been known for some time and Zwartouw and Westwood (1958) and Paul (1959) have pointed out it is of obvious value in adjusting the medium to physiological pH. The mechanism by which external pH influences glycolytic rate is however by no means clear. Furthermore, increase in glycolysis with pH cannot be considered except in conjunction with the associated effects of pH on assimilation and respiration. The finding in the present study that the respiration rate of glucose is unaltered between pH 6.6 and 7.9 despite the marked change in the rate of glycolysis is in general agreement with Phillips and Andrew (1960). Danes and Paul (1961) too have observed that cells grown at pH 7.4 do not show changes in respiration rate if this is measured between pH 6.6 and 7.8 soon afterwards. Tables 13 and 14 show that glucose incorporation is significantly reduced at high and low pH values although respiration rate is virtually unaffected.

At present no one hypothesis seems adequate to account for the divergent behaviour of such characteristically interdependent processes as respiration, glycolysis and assimilation with pH. Moreover the question of pH control of mammalian cell metabolism has not been discussed to any extent; a situation which reflects the lack of conclusive information on several fundamental questions. It is proposed therefore to outline the general problems posed by the experimental findings and discuss them in what is inevitably a speculative manner.

Possibly the most critical question is whether the metabolic changes observed on altering external pH are mediated through changes in the pH values within the cell. The fact that glucose respiration is unaltered between pH 6.6 and 7.9 suggests that pH within the cells at the sites of respiratory metabolism remains fairly constant over this range; it seems unlikely that the resultant of the kinetics of the enzyme systems involved would be unaffected if this were not so. Certainly this is indicated by work on sub-cellular fractions such as mitochondrial enzyme systems (Cooper and Lehninger, 1956) and isolated mitochondria (Chance and Conrad, 1958) which typically show decided reaction optima in the region of pH 7.0 with substantial inhibition at higher and lower values. This evidence is however circumstantial at best.

A vast literature exists on the general problem of pH values within cells. The term "cytoplasmic pH" is of doubtful significance: variations of pH within the cytoplasm and between locations of specific enzymic activity must exist, and cytoplasmic pH measured conventionally merely indicates a resultant of these. Methods used to determine this include the use of vital indicator dyes (Rous, 1925), measurement of fluid removed from cells (Bodine, 1926), microinjection of sulphonated acid dyes (Chambers, Pollack and Hiller, 1927) and potentiometer measurements of single cells (Caldwell, 1954, 1958). All of these have obvious and serious defects, nevertheless it is important that most measurements of mammalian cells indicate pH values on the acid side of neutrality. Chambers and Chambers (1961) cite a very large number of microinjection determinations on such diverse animal tissues as echinoderm eggs, frog ova, epithelium, intestinal mucosa, liver, pancreas and mammary carcinomas and sarcomas grown in tissue culture, all of which lie within the range 6.8 ± 0.2. Caldwell (1956) using a glass electrode recorded the internal pH of the crab muscle fibre as 6.9. Calculations by Hill (1955) using the Henderson-Hasselbach equation

and the experimentally determined carbon dioxide tensions of muscle and medium, indicated an intracellular pH of 6.75 for rabbit muscle. On the same principle, Wallace and Lowry (1942) calculated 6.9 for muscle fibres. More recently Kotsyuk and Sorokina (1961) using very fine electrodes, found frog sartorious muscle fibres to be in the region of 7.0.

Little is known of the buffering capacity of cytoplasm, although early experiments by Reznikoff and Pollack (1928) and Pollack (1928) purported to show that amoebae could control small local changes in pH induced by microinjection of phosphate buffers and 0.01 N HCl. In general the experiments described indicate that the isoelectric points of cytoplasmic proteins lie on the acid side of pH 7.0, and presumably exert a buffering effect there.

Recently Mitchell (1961) has shown that the osmotic barriers of bacteria and to a lesser extent mitochondria resist the passage of protons. Hill (1955) concluded that the intracellular pH of frog muscle fibres was characterised by stability to changes in external pH. Kotsyuk and Sorokina (1961) found that external values of 5.0 and 10.0 produced only very slow changes in intracellular pH (7.0) in the direction of the external pH, until new steady state levels were established. Changes were more obvious in the case of external alkaline pH values, but still small.

From these considerations it seems reasonable to suppose that if the L cell follows the pattern of other mammalian cells, its resultant internal pH is (i) probably on the acid side of 7.0, (ii) maintained to a large extent against external pH fluctuations of 6.6 to 7.9. Considerable evidence exists that some metabolic process is essential for the maintenance of pH within cells. The most conclusive is that of Hill (1955), Caldwell (1956, 1958), Kotsyuk and Sorokina (1961) and others who have shown that the observed pH shifts in muscle fibres in response to various external pH values, fall far short of those

expected if the distribution of protons across the membrane were governed on a purely physical basis such as Donnan's equilibrium. Without prejudice as to the nature of the mechanism involved, it follows that the work done must be a function of the difference between intra- and extracellular pH and involve the hydrolysis of ATP.

It is therefore suggested that maintenance of the internal environment of L cells against unsuitable external pH involves hydrolysis of ATP. That pH 7.9 is very far removed from the optimal physiological pH is shown not only by decrease in 14-C glucose assimilation in these cells but by Paul's (1959) report that growth is reduced by high pH. In this respect, the effects of pH 7.9 on membrane integrity and the general ionic content of the cell are unknown but may well be substantial, requiring increased metabolism to maintain the internal status quo.

In view of the conclusion that glycolysis is limited by the availability of ADP, it might be supposed that any process increasing the hydrolysis of ATP within certain limits would lead to an increased rate of glycolysis. It is of interest that membrane ATPase systems have recently been suggested as important regulators of glycolysis. These systems, concerned in the transport of ions, must be regarded as of ubiquitous occurrence and of fundamental importance in the maintenance of the internal environment. Whittam, Ager and Wiley (1964) have shown that lactic acid production in non-respiring erythrocytes is inhibited by inhibitors of membrane ATPase and conclude that this is due to a decrease in the rate of hydrolysis of ATP and therefore the availability of ADP for phosphorylation by 1:3-diphosphoglycerate. Recent work by Jones, Norris and Landon (1963) is of relevance. These authors showed that addition of membrane preparation containing ATPase stimulates the glycolytic activity of rat kidney homogenates more than 50% by increasing the 3-phosphoglycerate kinase reaction, through hydrolysis of ATP associated with this system. Since this indicates a direct coupling of a metabolic

reaction which generates ATP in the cytoplasm with a membrane ATPase which participates in the active transport of sodium and potassium, the authors suggest that this supports evidence (Wu and Racker, 1957) that glycolytic ATP is used for transport processes. Further evidence is required to justify this conclusion, and with the isolation of a membrane fraction showing high ATPase activity from L cells (Sinclair, 1964) work is now proceeding in this laboratory to investigate certain aspects of the problem.

A speculative hypothesis on the action of high pH on L cells may be suggested then: that processes concerned in the maintenance of the internal environment are activated with an increase in the rate of ATP hydrolysis and consequently the availability of ADP to the glycolytic system which is stimulated as a result. Despite the very significant increase in glycolysis, the rate of ATP production is only 7.5 s.d. 1.7% greater at pH 7.9 than at pH 7.4. This does not appear to be unreasonable. The problem is however, complex, and information is required on many fundamental aspects of cell physiology before any acceptable theory can be formulated. For example, the decrease in 14-C glucose assimilated at high external pH is not easy to reconcile with the idea that the pH values within the cell are maintained at their physiological optimum.

(iii) THE EFFECTS OF 2:4-DNP ON GLYCOLYSIS, GLUCOSE INCORPORATION, AND AIB UPTAKE UNDER ANAEROBIC CONDITIONS

In agreement with the findings of Warburg (1956) and Phillips and Feldhaus (1956) on L cells, a very marked Pasteur effect was observed. At pH 7.4 the rate of anaerobic glycolysis was 3.1 to 3.8 times that of aerobic glycolysis. The effect of hydrogen ion concentration was similar to that on aerobic glycolysis, lactic acid production increasing greatly with pH. Anoxia, however, clearly abolished the apparent block to lactic acid production under aerobic conditions at pH values less than about 7.0. At pH 6.3 substantial anaerobic glycolysis occurred, although much less than at higher pH.

The theory developed by Racker (1956), Chance and Hess (1956) and others is still the most acceptable explanation of the Pasteur phenomenon namely, that it is the direct consequence of ADP and Pi no longer required for respiratory chain phosphorylations becoming available for substrate level phosphorylations. The mechanism proposed for the Pasteur effect is therefore basically similar to that postulated for the increased glycolysis induced by 2:4-DNP in respiring cells (page 123). There is however a considerable difference between the rate of anaerobic glycolysis and the rate of 2:4-DNP stimulated aerobic glycolysis, the maximum rate of the latter being up to 1.5 times that of the former. Under the experimental conditions used there is no reason to postulate that anaerobic glycolysis is limited by anything other than the availability of ADP. Table 21 shows in fact that it can be stimulated by 2:4-DNP. Stimulation of anaerobic glycolysis by addition of uncouplers has from time to time been reported. For example, frog muscle (Greville, 1959) and certain ascites cells (Emmelot and Bos, 1959) respond to 2:4-DNP in this manner, and dinitrocresol has been found to increase anaerobic glycolysis of sarcoma 180 cells 1.7 fold (Clowes and Keltch, 1954).

Emmelot and Bos (1959) showed conclusively that ADP and Pi could substitute for 2:4-DNP in increasing the anaerobic glycolysis of cell homogenates, and suggested that the action of the uncoupler in increasing anaerobic glycolysis in whole cells was due to its stimulation of ATPase activity, thereby increasing the availability of ADP for phosphorylation. The findings on L cells are most reasonably explained in these terms. This implies that the glycolytic capacity is so high that it is unsaturated even under anoxia when increased levels of ADP and Pi are available for substrate level phosphorylations. 2:4-DNP is considered to increase ATP hydrolysis and ADP availability, thereby establishing a new steady state rate of anaerobic glycolysis. Although a significant stimulation of anaerobic glycolysis by 2:4-DNP has been shown for L cells, the extent was variable even under carefully controlled conditions. The reason for this is obscure. It should be noted, however, that the uncoupler was always added after a substantial gassing period in the absence of exogenous substrate. During this period some lactic acid was always produced from endogenous substrate (page 112) and subtracted from the final result. Quantities of lactic acid produced in this way seldom varied by more than 10 per cent but it seems likely that small variations in endogenous substrate during gassing might lead to differences in the adenosine nucleotide levels, which on tipping 2:4-DNP and glucose could be amplified into substantial differences in glycolytic rates. In no case did 2:4-DNP stimulated anaerobic glycolysis reach the rate of 2:4-DNP stimulated aerobic glycolysis. This may be a consequence of a low initial ATP level, and therefore greater competition for this between the kinases and stimulated ATPase system or systems.

2:4-DNP notably decreased the incorporation of 14-C glucose by cells under anaerobiosis. Combining information from Tables 21 and 26 it can be calculated that the mean quantities of 14-C glucose assimilated per u mole ATP produced per hour were - 1.83 ug. (no uncoupler), 1.02 ug. (0.1mM) and

0.92 ug. (0.2mM). This means that the extent of synthesis by 2:4-DNP treated anaerobic cells cannot be equated with the theoretical net production of ATP. Indeed the inference is that much less rather than more of the stimulated ATP production is available for synthesis. These findings are in general support of that of Emmelot and Bos (1959) who found that S_3A ascites cells showing a nearly two-fold rate of anaerobic glycolysis with 0.1mM 2:4-DNP only incorporated leucine at 50% of the normal anaerobic rate. They are in contrast to the report by Robinovitz and Olsen and Greenberg (1955) that 2:4-DNP was a poor inhibitor of the incorporation of amino acids into Ehrlich ascites cells under conditions of anaerobic glycolysis.

Graph 25 and Table 27 show that there is a substantial net uptake of 14-C AIB under anaerobic conditions. Where glucose is present as substrate, this uptake does not appear to be closely linked to net ATP production. For example, uptake under anaerobic conditions is in the region of 56 per cent of that under aerobic conditions, although net ATP production is only 20 per cent of the aerobic rate. Under endogenous anaerobic conditions, net AIB uptake was still approximately 40 per cent of that under aerobic conditions with glucose present, and even using glycolytic inhibitors Iodoacetate and arsenite as well, the AIB uptake was no less than 40 per cent. These findings were unexpected and destroy the assumption that AIB uptake is solely an energy requiring process. This matter is further discussed later (page 147). A finding of some import is that 2:4-DNP caused a notable decrease in net AIB uptake under anaerobiosis with glucose present. As in the case of 14-C glucose incorporation therefore, the small increases in anaerobic glycolysis induced by 2:4-DNP are not reflected in concomitant increases in rate of AIB uptake. These findings are in agreement with the idea that a consequence of ATPase stimulation is that competition between kinases, synthetic and transport reactions, and ATPase systems for ATP is intensified, and results in a new equilibrium in which the rates of synthesis and transport at least are

decreased.

(iv) THE EFFECTS OF 2:4-DNP UNDER AEROBIC CONDITIONS

There can be no doubt that the changes in metabolism, synthesis and transport observed on addition of 2:4-DNP to L cells are principally due to its action as an uncoupler of oxidative phosphorylation. The extent of uncoupling at any of the concentrations used is, however, uncertain, as uncoupling can only be determined by measurement of P/O ratios. Calculations made for intact cells by measuring changes in the inorganic phosphate of the medium during incubation are obviously unreliable. Furthermore, even if the practical problem of obtaining sufficient fresh mitochondria at one time could be overcome, the dose/response information obtained for these could not be confidently applied to mitochondria in vivo for several reasons. For example, the structure and physiology of the plasma and the mitochondrial membranes are by no means synonymous, the equilibria for the uncoupler across these membranes are not known, the problem of whether the active form is the ion or the undissociated molecule or both has not yet been resolved, and there is lack of information on the microenvironment of the mitochondrion and possible fluctuations of pH with the physiological state of the cell. At present, therefore, 2:4-DNP modified P/O ratios cannot be critically determined for systems as complex as intact mammalian cells. Consequently, turnover of ATP in 2:4-DNP-treated cells is hazardous to calculate. The data from normal metabolism is however amenable to calculation making the usual assumptions (page 75). The rate of synthesis of ATP by control cultures in the six experiments at pH 7.4 depicted in Graphs 19 and 20 is 5.65, s.d. 0.14 u. moles per mg. protein hour. Since the total adenine nucleotide content is of the order of 0.03 u moles per mg. protein (page 118) it may be inferred that the total nucleotide can be turned over in about 19 seconds. This assumes that all the nucleotide is available for metabolism. This rate is somewhat faster than that of 30 seconds calculated by Schmid (1957) and Ibsen, Coe and

McKee (1958) for ascites tumour cells, but not unreasonably different considering divergences in size, metabolism and growth conditions. It indicates that fundamental changes in the phosphorylation cycle, such as uncoupling of oxidative phosphorylation, could very rapidly be reflected in changes in the nucleotide ratio and cellular processes directly or indirectly dependent upon ATP or ADP. In fact this has been shown to be the case in the present study.

It has been noted that during glucose metabolism under manometric conditions, approximately 90% of the total adenine nucleotides in the cell is in the form of ATP, about 10% as ADP and that only trace quantities of AMP are present. (It must be stressed that this pattern observed under manometric conditions need not reflect steady state nucleotide proportions in exponentially growing cells when transport and synthesis almost certainly proceed more rapidly.)

The addition of 2:4-DNP causes significant changes in the proportions of the adenine nucleotides, and has been carefully examined at three particular concentrations - 0.05, 0.10 and 0.20mM. In each case a new steady state pattern was established within fifteen minutes of addition of the uncoupler, and possibly sooner. The establishment of such equilibria by the action of 2:4-DNP at these concentrations were accompanied by rapid changes of metabolic and synthetic processes to new and constant rates which again depended upon constant external conditions. For example, on adding 2:4-DNP the onset of stimulation or inhibition of glucose respiration was rapid. Although the time required for temperature equilibration after tipping obscured precise determination of this, the new rates reached steady values within a few minutes. Ibsen, Coe and McKee (1958) using an oxygen electrode, noted a short lag phase of 2 - 4 minutes before the uncoupler exerted its full effects on Ehrlich ascites cells, possibly due to low permeability of the membrane. Other authors (Simon, 1953) have commented on the rapidity with which new steady respiration

rates are established. While glycolytic rates could not be followed as carefully as respiration, the results indicate a similar rapid transition to new steady rates. Experiments on the rates of incorporation of 14-C glucose into acid precipitable cellular components show that the 2:4-DNP inhibited rates were established quickly and remained relatively constant for an hour and a half at least. The rate of uptake of 14-C AIB was rapidly altered by 2:4-DNP but as discussed later (page 148), interpretation of the subsequent rates of uptake is difficult as factors other than ATP dependent processes are involved.

The addition of 2:4-DNP at these concentrations to intact L cells is therefore followed by the rapid establishment of new steady state levels of adenine nucleotides, and new and constant rates of respiration, glycolysis and synthesis. As Mitchell (1961) has remarked, in the exact sciences cause and effect are no more than events linked in sequence. Some of the principles involved in the establishment and maintenance of equilibria between these systems will now be considered.

The effects of 0.05mM 2:4-DNP

Addition of 0.05mM 2:4-DNP to cells metabolising glucose, produced increases of 115% in the respiration rate and about 200% in the rate of glycolysis. Dodds and Greville (1934) working on slices of rat sarcoma were among the first to show that the dinitrophenols could influence aerobic glycolysis as well as respiration. At that time the phenomenon of simultaneous stimulation of respiration and glycolysis was a new and entirely unsuspected one, contrasting with the findings of Pasteur that glycolysis could be stimulated at the expense of respiration, and those of Barron and his colleagues (e.g. Barron and Harrop, 1928) that the action of methylene blue in stimulating respiration was in many cases correlated with a simultaneous decrease in glycolysis. With the finding that 2:4-DNP uncoupled oxidative phosphorylation (Loomis and Lipmann, 1948) and the development of the theory of competition between respiration and glycolysis for Pi and ADP as a regulatory factor for cell metabolism (Lynen, 1941,

1958; Racker, 1956; and others) an obvious hypothesis for the action of 2:4-DNP was formulated, namely that increased respiration was the result of releasing oxidations from the rate limiting phosphorylations, and increased glycolysis was consequential to the ADP no longer being depleted by respiratory chain phosphorylations becoming available for substrate level phosphorylations in the cytoplasm. A large body of evidence indicates that the theory of competition and its release by uncoupling is substantially true for many tissues. The work of Gatt, Krimsby and Racker (1956) has already been mentioned. These authors showed that reconstituted glycolytic enzyme systems competed with respiring mitochondria for ADP, and that 2:4-DNP or the omission of a glycolytic enzyme released this competition. Emmelot and Bos (1959) similarly showed that glycolysis of ascites carcinoma homogenates was markedly dependent upon the concentrations of Pi and ADP, depending upon experimental conditions. When these were available in excess, no stimulation by 2:4-DNP could be induced.

An important feature of the new steady state concentrations of the adenine nucleotides accompanying L cell metabolism in 0.05mM 2:4-DNP is that at 20% of the total, ADP is present at twice its control value. Measurable quantities of AMP are also present amounting to about 5%. In contrast, ATP at 75% of the total adenine nucleotides, is about 20% less than in the absence of uncoupler. These findings taken with the increased rates of respiration and glycolysis indicate that substantial uncoupling occurs at 0.05mM 2:4-DNP concentration: the sequence of events being envisaged as: (a) stimulation of respiration as oxidations are released from the rate limiting phosphorylations, (b) a rapid build-up of ADP at the sites of oxidative phosphorylation followed by release into the extramitochondrial cytoplasm, (c) an increase in the rate of aerobic glycolysis as a result, (d) the attainment of a new steady state pattern of metabolism; phosphorylation and dephosphorylation being balanced at the equilibrium which is thermodynamically most favourable.

Complete uncoupling is unlikely at 0.05mM 2:4-DNP; the respiratory rate is approximately 10% less than the maximal rate induced by about 0.075 mM uncoupler, and lactate production is 50% less than its maximum at about 0.20mM 2:4-DNP. Increases of this nature can scarcely be envisaged if uncoupling is complete at 0.05mM. A further argument against complete uncoupling at this concentration is that if this were so, the theoretical rate of ATP production calculated from substrate level phosphorylations alone (Graphs 19 and 20) would be 2.45 s.d. 0.12 u moles ATP per mg. protein hour. This is merely 43% of the theoretical rate of ATP synthesis of coupled cells and seems inadequate to support a constant rate of 14-C glucose incorporation averaging 74% of the control rate. Furthermore, since it has been shown (Table 31) that 2:4-DNP increases the ATPase activity of homogenates, if complete uncoupling were assumed the effective production of ATP for synthesis and metabolism by substrate level phosphorylations would be even less than 2.45 u moles per mg. hour.

It is well known that the carbon atoms from glucose are assimilated into most cell fractions - lipid, nucleic acids and protein as well as carbohydrate (Crockett & Leslie, 1963). The incorporation of 14-C glucose is therefore a useful index of general synthetic activity which appears to be proceeding too rapidly for the calculated substrate level phosphorylations at this concentration of 2:4-DNP.

The Effects of 0.10mM 2:4-DNP

The addition of 0.10mM uncoupler to cells metabolising glucose resulted in respiration rates approximately the same as that induced by 0.05mM - about 115% greater than that of control cells. In this case however the rate was 10% depressed relative to the maximal rate at about 0.075mM. The glycolytic rate showed an average increase of 320% over the control rate and was therefore considerably greater than the rate at 0.05mM, although about 23% lower than

the maximum rate induced by 0.20mM uncoupler. Since the rate of glucose utilisation from the medium in the six experiments at pH 7.4 (Graphs 19 and 20) was 14.4% (s.d.0.9) greater at 0.20mM 2:4-DNP than at 0.10mM ($P<0.01$) it is clear that the increased lactate production at the higher concentration is not merely a result of pyruvate which entered the respiratory cycle at 0.1mM uncoupler being reduced to lactate due to respiratory inhibition at 0.20mM. At 0.10mM 2:4-DNP therefore, the rate at which glucose is metabolised is submaximal.

Although it has already been stressed that the extent of uncoupling is uncertain at any of the 2:4-DNP concentrations used, it is reasonable to suppose that at 0.1mM uncoupling is virtually complete. Substantial evidence for this is that the pattern of inhibition of 14-C glucose incorporation with increasing uncoupler concentrations changed abruptly at 0.1mM. Table 25 shows that an average inhibition of 70% s.d. 3.3 occurred at this concentration with relatively little further inhibition at much higher concentrations. For example, at 0.5mM the inhibition only increased to approximately 83%. A very similar pattern was seen for 14-C AIB uptake (Graph 25) although in this case the inhibition of net uptake was much less severe. In L cells therefore under these conditions there is relatively little further inhibition of energy requiring processes at concentrations of uncoupler greater than 0.10mM. A further argument in favour of maximum uncoupling at this concentration is that Hemker (1964) has recently shown that this invariably occurs for mitochondria at concentrations of dinitrophenols which induce maximum respiratory stimulation. As already noted, 0.10mM 2:4-DNP is greater than the concentration (0.075mM) inducing this in intact L cells at pH 7.4. It is suggestive too, that many authors have shown respiratory chain phosphorylations in sub-cellular systems to be completely uncoupled by 0.1mM 2:4-DNP (Kirpekar and Lewis, 1960; Slater and Lewis, 1954) although this need not necessarily apply to intact cells.

Calculations from the respiration and glycolytic rates induced by 0.1mM 2:4-DNP (Graphs 19 and 20) show that the theoretical ATP production from substrate level phosphorylations is 3.10 s.d. 0.13 u moles ATP per mg. protein per hour. This is a 45% reduction from the rate in control cells calculated from both respiratory chain and substrate level phosphorylations. As already stated, the average inhibition of 14-C glucose incorporation at 0.1mM 2:4-DNP is 70%. On the basis of the information in Table 25 an average of 1.35 ug. 14-C glucose is incorporated per mg. protein to every 1 u mole ATP produced in coupled cells; the comparable figure for cells at 0.1mM 2:4-DNP assuming complete uncoupling is 0.74 ug 14-C glucose. The cost of 14-C glucose incorporation in terms of ATP production would therefore appear to be greater in uncoupled cells. Furthermore, if uncoupling were in fact incomplete at 0.1mM 2:4-DNP the cost of incorporation would be even greater at that concentration.

Consideration of the relationship of 14-C AIB uptake to ATP production shows a somewhat similar situation. Graph 25 shows that the net uptake over 10 minutes was decreased by approximately 35.5% at 0.1mM, 38% at 0.2mM and about 45% at 0.5mM uncoupler, the AIB being added 10 minutes after the uncoupler and glucose. Since at 0.1mM the theoretical ATP yield was 45% lower than in coupled cells (the presence or absence of 14-C AIB in the suspension media makes no significant difference to the rates of respiration and glycolysis with or without uncoupler) these figures might be taken to indicate that net AIB uptake is less inhibited by 0.1mM uncoupler than glucose incorporation, and correlates well with net ATP yield. The effects of anoxia on cells lacking exogenous glucose with and without glycolytic inhibitors, indicates however that the net uptake of AIB is not due solely to metabolic energy. Table 27 shows that the net uptake by anaerobic cells without external substrate and poisoned with Iodoacetate was fully 40% of the uptake by aerobic glucose respiring cells without inhibitor. Furthermore, this uptake under

maximum respiratory and glycolytic inhibition was concentrative, and the most reasonable explanation is that it occurs by exchange diffusion, the extra-cellular AIB exchanging with one or more internal amino acids. This phenomen is well documented (Heinz and Waller, 1958; Christensen, 1960). No "uphill" transport is involved and the energy inherent in the pre-established amino acid gradient is preserved. Circumstantial evidence for its occurrence in this case is that extracellular glycine can markedly displace 14-C AIB from preloaded cells. An alternative hypothesis is that uptake under conditions where the energy metabolism is inhibited maximally is mediated by the energy inherent in the asymmetry of potassium across the membrane (Christensen, Riggs, Fischer and Palatine, 1952; Riggs, Walker and Christensen, 1958; Christensen, Riggs and Coyne, 1954). Assuming that the uptake under these conditions is, in fact, by exchange diffusion, the inhibitory effects of 2:4-DNP on the remaining component of uptake are rather greater than on net uptake. Taking the uptake by anaerobic endogenous IOD poisoned cells as a baseline, 0.1mM 2:4-DNP inhibits the remainder by approximately 60%.

In a recent paper, Riggs and Walker (1963) concluded that in Ehrlich ascites cells the transport and incorporation into protein of 14-C amino acids were within certain limits, independent processes in that either could be altered without an apparent effect on the other. For example, pyridoxal was found to inhibit incorporation and stimulate transport at the same time, while exogenous glucose increased incorporation without affecting transport rate. It was concluded that incorporation was typically inhibited to a greater extent than transport by conditions interfering with energy metabolism. Interpretation of the results of these experiments is difficult because of the inherent problems of following amino acid metabolism within the pool - transamination, deamination and subsequent respiration, synthesis of non-essential amino acids and the kinetics of dilution of cold intracellular amino acids by

14-C homologues, in general, require assumptions which make the specific activity of cell protein difficult to assess with confidence. In addition, active transport of amino acids was assumed throughout. Ellis & Scholefield (1961) examined the effects of uncouplers on the uptake and incorporation of glycine by tumour cells, and concluded that DNP inhibited incorporation more than uptake. In this case no investigations were made on the nature of the uptake. The present findings on AIB uptake and glucose incorporation by L cells support the conclusion that total AIB uptake is much less sensitive to inhibition of ATP production than glucose incorporation. The postulated ATP dependent component of AIB uptake, however, does not differ substantially from glucose incorporation in its response to the lowering of ATP production by 0.1mM 2:4-DNP.

At this concentration of the uncoupler both incorporation and transport are reduced to a greater degree than might be anticipated on the basis of the theoretical ATP yield from substrate level phosphorylations. Several alternative hypotheses might account for this - (i) ATP produced by substrate level phosphorylations may be less available to the sites of transport and synthesis. This seems unlikely. In the present experiments, the postulated ATP dependent transport of AIB by anaerobic cells is reasonably proportional to the ATP produced, on comparison with that of aerobic cultures. For example, the mean uptake is about 27% that of aerobic cells, although the ATP production is only 20%. Furthermore, growth of mammalian cells in culture under anaerobic conditions has been reported on several occasions (Dales, 1960; Harris, 1956; Jones and Bonting, 1956). Johnston (1959) states that anaerobic glycolysis in Ehrlich cells can support high rates of alanine and glycine uptake. Rabinovitz, Olson and Greenberg (1955) concluded that anaerobic glycolysis supported rates of incorporation of amino acids into proteins comparable with those achieved under aerobic endogenous conditions.

Emmelot and Nout (1959) produced evidence that glycolytic ATP had ready access to the acetate activating enzyme. Selvarj and Sbarra (1964) noted that anaerobiosis did not inhibit phagocytosis of white blood cells. It would appear from these and other reports that ATP produced by substrate level phosphorylations can readily participate in most cellular processes.

(ii) One possibility is that with a substantial decrease in ATP production, utilisation of ATP may be on the priority basis with the maintenance of the integrity of the limiting membrane and the asymmetric distribution of ions across it of greater importance than synthetic processes. An argument against this operating in this case is again that decrease in ATP production by anoxia does not inhibit AIB uptake to any greater degree than expected. Furthermore, Dales (1960) has calculated that growth of L cells under anaerobic and aerobic conditions is proportional to the theoretical ATP yield in each case, although under anaerobic conditions this was only about one third of that under aerobic.

(iii) A more plausible explanation is that a proportion of the ATP synthesised by substrate level phosphorylation is competed for and hydrolysed by a 2:4-DNP stimulated ATPase system, and therefore lost for synthetic processes. Certainly it has been shown that the uncoupler stimulates ATPase activity in cell homogenates, and its effects of stimulating anaerobic glycolysis and inhibiting 14-C glucose uptake under anoxia indicate that ATP produced by substrate-level phosphorylations is accessible to the site of activity.

The Effects of 0.2mM 2;4-DNP

Addition of 0.2mM uncoupler to L cells induced steady respiration rate which was about 45% greater than that of control cells, but over 30% lower than the maximal rate induced by about 0.075mM uncoupler. Inhibition of respiration by high concentrations of uncoupler has been observed before for micro-organisms and plant cells as well as animal cells (Simon, 1953).

Shacter (1955) suggested that a change in the integrity of the plasma membrane may be one reason for this, and noted that both Pi and esterified P leaked from such cells on washing. The constancy of respiration rates with time and the absence of significant reductions in total adenosine nucleotides in the experiments described with L cells at 0.2mM 2:4-DNP suggests that the membranes are not severely or progressively damaged under these conditions. Several authors have in fact shown that high concentrations of substituted phenols inhibit isolated enzyme systems including cytochrome reductase and the flavoproteins. (Krahl, Keltch and Clowes, 1940; Haas, Harrer and Hogness, 1942). Recently, however, Hemker (1964) has shown than alkyl-dinitrophenols inhibit succinate, glutamate and B-hydroxybutyrate oxidation by intact mitochondria at concentrations which have no effect on succinate and NADH dehydrogenase or cytochrome c oxidase activities. He concluded that the site of inhibition is located between NADH and flavoprotein, and between flavoprotein and cytochrome c. The third phosphorylating site between cytochrome c and oxygen was not inhibited although readily uncoupled. The mechanism of inhibition suggested by these experiments was that excess of the uncoupler (Ø) bound a relatively large amount of the unknown mediator I in the oxidative phosphorylation mechanism proposed by Slater, in which case the bound I is not available for reaction (1) which therefore proceeds more slowly. This seems a reasonable hypothesis to account for the marked inhibition of respiration in L cells at 2:4-DNP concentrations which produced no inhibitory effects on glycolysis.

$$AH_2 + B + I \rightleftharpoons A\sim I + BH \quad (1)$$

$$A\sim I + \phi \rightleftharpoons I-\phi + A \quad (2)$$

$$I-\phi \rightleftharpoons \phi + I \quad (3)$$

0.2mM 2:4-DNP is of further interest in that aerobic glycolysis was stimulated maximally by this concentration to a rate which was almost six times that of control cells. As already discussed, despite the marked reduction in

respiration rate, the rate at which glucose was metabolised was also maximal and significantly greater than at 0.1mM. This is interesting in view of the suggestion made earlier that uncoupling was virtually complete at 0.1mM. Consideration of the steadystate ratios of ATP:ADP:AMP in cells treated with 0.1mM and 0.2mM (66:26:8 and 56:32:12 respectively), show that a further decrease has occurred in ATP with subsequent increases in ADP and AMP at the higher uncoupler concentration. Although the maximum rate of aerobic glycolysis is found at this point, this does not imply that the glycolytic enzyme system is saturated; the rate limiting factor may now be the availability of ATP for kinase activity.

It seems inevitable that the higher glycolytic rate at 0.2mM 2:4-DNP relative to that at 0.1mM is related to the change in the proportion of the nucleotides. Three factors may be of relevance - (i) It is possible that, despite the evidence to the contrary, uncoupling was in fact incomplete at 0.1mM and with further uncoupling at 0.2mM more ADP has become available; (ii) A further possibility is that ADP involved in the substrate level phosphorylation of the succinyl co-A to succinate step in the TCA cycle at 0.1mM has become available to the glycolytic pathway as a result of the inhibition of the oxidative cycle by 0.2mM; (iii) The basis of the differences in the metabolic patterns induced by 0.1mM and 0.2mM 2:4-DNP may be increased ATPase activity at the higher concentration.

No firm conclusions can be drawn on whether one or all of these principles are involved. It seems likely that the latter is of some importance. In this respect the maximum ATPase activity in L cell homogenates was induced by 0.2mM 2:4-DNP, precisely the concentration which induced maximum aerobic glycolysis in intact cells. Furthermore, several reports have shown that the maximum ATPase activity of mitochondria is induced by considerably greater concentrations of uncoupler than are required to bring about complete uncoupling (Kirpekar and Lewis, 1960; Hemker, 1964). The theoretical net

ATP yield at 0.2mM uncoupler concentration from substrate level phosphorylations is only in the region of 5% greater than at 0.1mM since the increase in cytoplasmic phosphorylations is counteracted to some extent by decrease in the TCA cycle substrate level phosphorylation. Conversely both 14-C glucose assimilation and AIB uptake show small decreases, emphasising further that with increasing uncoupler concentrations, decreasing quantities of the theoretic substrate level ATP yield are available for these processes.

* * * * *

The most important general finding in this study is the ability of L cells to respond to fundamental alteration of their phosphorylating cycle by the rapid establishment of new steady state rates of metabolism and synthesis. The central factor in this is the high glycolytic potential of the cell which enables a considerable proportion of the normal ATP production to be provided by substrate level phosphorylations. A further feature of interest is the extent to which the oxidative cycle can be stimulated. Considering the carefully controlled growth conditions and the relative constancy of respiration and glycolytic rates under these circumstances, it is of interest that these can be immediately stimulated to about 2.5 and 6 times their normal and possibly more.

(v) THE EFFECTS OF pH ON UNCOUPLING ACTIVITY

Graphs 19 and 20 show that the action of 2:4-DNP on respiration and glycolysis is greatly modified by pH. Between pH 6.6 and 7.9 increasing 2:4-DNP concentrations produce the same characteristic effect on respiration and glycolysis, namely stimulation to a maximum rate followed by depression at higher concentrations. Approximately 3 times more uncoupler is required at pH 7.9 to maximally stimulate respiration and glycolysis than at pH 6.6.

This underlines the importance of controlling the pH when evaluating the effect of a given concentration of 2:4-DNP on a biological system. Failure to take this into account has led to errors in the past, the conclusion of Myers and Slater (1957) discussed on page 27 being one example.

The findings show that the maximum stimulation of respiration and glycolysis by 2:4-DNP is of the same order, between pH values 6.6 and 7.9, although much larger quantities of uncoupler are required at the higher pH. The fact that the rate of glycolysis at pH 6.6 can be stimulated to this extent supports the idea already expressed that inhibition at this pH in the absence of uncoupler is not due to a direct inhibitory effect of pH on any enzyme system.

As the pK for 2:4-DNP is 4.4, decreasing pH over the physiological range increases the concentration of the undissociated molecule. The increasing effectiveness of 2:4-DNP with decreasing pH appears therefore to be related to the increasing concentration of the undissociated molecule in the medium. Insufficient data is available to analyse the nature of this relationship. Simon and Beevers (1952) who investigated the effects of pH on the action of nitrophenols, hydrogen fluoride and other weak acids relative to yeast respiration and fungal growth concluded that both undissociated molecules and, to a lesser extent, ions were active. Hemker (1964) has produced evidence that for any given alkyldinitrophenol it is the concentration of the

undissociated phenol in the medium which determines the degree of uncoupling and proposes that it must be taken into the mitochondrial lipid before it can act.

In terms of the Meyer-Overton liphoidtheory (Höber, 1945) undissociated molecules penetrate biological membranes more readily than ions. It may be concluded from the rapid attainment of steady state respiration at the pH values tested that the effect of pH on the rate of penetration of 2:4-DNP into L cells is of less importance than the conditions prevailing when steady state has been reached.

(vi) THE EFFECTS OF SODIUM SALICYLATE ON RESPIRATION, GLYCOLYSIS AND 14-C GLUCOSE INCORPORATION

The addition of sodium salicylate to L cells results in changes in metabolism which indicate uncoupling of oxidative phosphorylation. These are simultaneous increases in the rates of respiration and glycolysis, and a decrease in 14-C glucose incorporation. Of these phenomena the best evidence for supposing that complete uncoupling occurs within the concentration range tested is that inhibition of 14-C glucose uptake is virtually complete at 7mM. The constancy of respiration rate with time at this concentration may be taken as indicating that the cells maintain their structural and functional integrity over the experimental period, and that the severe inhibition of incorporation is not due to cell necrosis. Accepting that uncoupling is probably complete in the region of 7mM, this differs from that induced by 2:4-DNP in that it is not accompanied by as marked increases in the rates of respiration and glycolysis. Several authors have observed this to be so for mitochondrial respiration. Kirpekar and Lewis (1960) have shown that in contrast to 2:4-DNP, the complete uncoupling of rat liver mitochondria by 10mM salicylate or less occurs with little change in respiration rate. Jeffrey and Smith (1959) observed that the respiration of mitochondria was scarcely affected by 2 - 5mM salicylate which caused 100% uncoupling of their system. Brody (1956) noted too that decreases in P/O ratio induced by salicylate were not accompanied by substantial increases in respiration.

The experiments with L cells show that the salicylate-stimulated rates of glycolysis are also substantially less than might be expected on the basis of the increases which accompany uncoupling by 2:4-DNP. The stimulation of respiration and glycolysis by the latter uncoupler has been explained primarily on the basis that these processes are normally limited by availability of ADP. Phosphate acceptor however cannot be limiting respiration and glycolysis in salicylate uncoupled cells. At present the most plausible explanation is

that the high concentrations of salicylate required for uncoupling inhibit the activity of enzymes of the oxidative and glycolytic pathways. A measure of evidence exists for this. Kaplan, Kennedy and Davis (1954) produced results which showed that salicylate at about 6mM concentration, inhibited a variety of oxidative enzymes of rat liver and kidney homogenate, especially succinic and a-ketoglutaric dehydrogenase. Recently Bryant, Smith and Himes (1963) have shown that 10mM salicylate inhibits malic dehydrogenase activity by 40% and 2mM inhibits isocitric dehydrogenase by 70% in vitro. Their results indicate that the mechanism of inhibition involves competition with the appropriate cofactor. Smith (1964) has proposed that any enzyme which requires a pyridine nucleotide cofactor may be inhibited by salicylate. This of course, includes several enzymes of the EMP, for example, lactic dehydrogenase and a-glycerophosphate dehydrogenase.

One consequence of the absence of substantial increases in respiration and glycolysis on uncoupling by salicylate is that the decrease in respiratory chain phosphorylations is not compensated for to any extent by increased substrate level phosphorylations. This difference between salicylate and 2:4-DNP seems to be reflected in the patterns of inhibition of 14-C glucose incorporation. With increasing concentrations of 2:4-DNP, 14-C incorporation is steadily inhibited until about 0.1mM, after which further inhibition is resisted probably as a result of substrate level phosphorylations. With increasing concentrations of salicylate, inhibition increases steadily until it is virtually complete, with no obvious resistant phase which could be interpreted as being due to substrate level phosphorylations.

DISCUSSION

GROWTH EXPERIMENTS

(i) ASPECTS OF GROWTH AND METABOLISM

L cells in modified WB 752/1 showed a pattern of growth and metabolism typical of mammalian cell cultures. Under standard conditions, cell growth was exponential for almost the whole 96 hours over which most experiments were conducted, with a mean generation time of about 45 hours. This is slightly less than that reported by most other authors for L cells in polymer-free defined medium, for example - 52 hours (Healey, 1955), 56 hours (Waymouth, 1959), 60 hours (Katsuta and Takaoka, 1960) and 44 hours (Fioramonti, Evans and Earle, 1956). Conversely, it tends to be rather longer than most mean generation times reported for growth in media containing serum, polymers such as PVP, and undefined nutrients like bactopeptone, for example - 32 to 43 hours (Waymouth, 1956), 35 hours (Katsuta et al, 1959), 23 hours (Swaffield and Foley, 1960) and 19 hours (Dales, 1960). Obviously little significance can be attached to comparison of growth rates from different laboratories with correspondingly different conditions of growth and certainly, in some cases at least, different sub-strains of L cells. In our laboratory, the addition of serum to WB 752/1 does not greatly increase the rate of proliferation, and apart from the nutritional complications which it imposes, its presence decreases the buffering power of the medium so that the pH falls much faster for the same initial rate of glycolysis.

During exponential growth, no significant differences have been found in the ratios of total ribose, desoxyadenosine equivalents and protein to one another or on a per cell basis. This is in agreement with the findings of Siminovitch, Graham, Lesley and Nevill (1957) and Hill, Bensch, Simbonis and King (1959) on suspension cells during exponential growth. Swaffield and Foley (1960) report changes in the content of nucleic acids and protein per cell during exponential growth of L cells but their data indicates that this phase was too short to justify

their conclusion. In fact, several authors including Paul (1959) and Mohberg and Johnson (1963) have stressed the variability of cellular components during lag and early stationary phases of growth.

Ruckley et al (1963) have pointed out the wide differences in dry weight reported by various authors for L cells. It would appear that a similar variance of other cellular components also exists in the literature at least. In the table below, the results of the present study are compared with figures taken from other authors' results for L cells in log growth phase.

pgm. per cell			
DNA	RNA	Protein	Authors
15	33	220	Siminovitch, Graham, Lesley and Nevill (1957)
19		250	Hill, Bensch, Simbonis and King (1959)
12	31	230	Paul (1959)
24	40	508	Swaffield and Foley (1960)
10 s.d.1	28 s.d.2	341 s.d.16	R E I D

The differences reflect not only growth conditions but different methods of assay also.

The pattern of glucose metabolism may be summarised. Glucose was used more rapidly during the first day than on subsequent days, the quotient decreasing by up to 50% over the experimental period of 4 days. The quotient for lactic acid produced, similarly decreased with time, and on the last day was less than half that on the first day. Typically, small quantities of α-keto acids appeared in the medium during the first day, increased slightly or maintained their level during the second day, and on subsequent days were removed from the medium, presumably by the metabolic activity of the cells. These findings therefore confirm the general pattern of glucose metabolism of growing cells reported elsewhere (Leslie, Fulton and Sinclair, 1957; Paul, 1959).

The decrease in acid production with time after renewal of the medium has been commented upon by several authors. Among the reasons suggested to account for this are (i) that the decreased rate of production is a consequence of a fall in external glucose concentration and the subsequent oxidation of an increasing proportion of the acids produced (Fulton, Sinclair and Leslie, 1956), (ii) that a Crabtree effect is involved (Rucker, Broda, Kellner and Frimmel, 1963), (iii) that the initial production of acid is largely a consequence of leakage until the external concentration approaches that normally existing in the cell (Leslie, Fulton and Sinclair, 1957), (iv) that a pH effect is important (Paul, 1959; Zwartouw and Westwood, 1958).

There can be little doubt that by varying the experimental conditions, each of those factors can be brought into operation to provide net results which are very similar. Under the described growth conditions, the availability of glucose is unlikely to be of importance since depression of the initial rate of glycolysis was apparent before the glucose concentration was appreciably depressed. Indeed, over the 96 hours, glucose in the medium only fell by about 20%. Furthermore, Munyon and Merchant (1959) investigating L cell growth in suspension, found that glycolysis was by no means closely related to the glucose concentration of the medium. Eagle et al (1958) found that for human liver cells the rates of utilisation of glucose and production of lactate were not appreciably influenced by glucose concentration between 20mM and 5mM. The results of manometric investigation on L cells (graph 15) show that under these conditions respiration and glycolysis were not affected until external glucose concentration fell below 5mM, a situation which never arose during growth. Suggestion (i) above is therefore thought unlikely.

Since there is no evidence that the initial glucose concentration depressed respiration or indeed that the decrease in glycolytic rate with time was accompanied by an increase in respiration rate, suggestion (ii) that a Crabtree effect is operating is rejected in this case.

As there is no evidence for a transport mechanism for lactic and α-keto acids (Giebel and Passow, 1960), it seems likely that their appearance in the medium is the result of diffusion down a concentration gradient (Fulton et al, 1957). It remains to be seen, however, whether the observed increase in the loss of acids on addition of fresh medium is the cause of increased glycolysis as apparently implied by Fulton et al (1956) or the effect. The former hypothesis necessarily means that the rate of the Embden-Meyerhof pathway is determined to a large extent by the intracellular concentrations of the acids, and that loss of these to the medium increases its rate. This appears to be altogether too fundamental a role to ascribe to products of metabolism which can readily pass through the membrane anyway (Hober, 1945), and of the several hypotheses on the control of glycolysis (page 129) has never been seriously considered. Zwartouw and Westwood (1958) found that if cells were maintained at pH 7.4, the presence of 20mM lactate in the medium did not inhibit either glucose utilisation, lactate production or cell multiplication.

It is suggested that the simplest explanation of the present results is that the rate of glycolysis during growth is primarily controlled by the direct effect of the pH of the cellular micro environment on metabolism, by a mechanism which is as yet unknown (pages 132-136). With the progressive formation of a confluent monolayer of cells under stationary conditions, the diffusion of carbon dioxide and acids from each cell must be considerably slower. Although over 4 days the decrease in pH of the bulk medium is only from 7.4 to 7.2 or 7.1, the differential between the initial and final pH of the cellular micro-environment is likely to be much greater due to the closer apposition of individual cell membranes.

An important feature of the present investigation is the constancy of the quotient "glucose unaccounted for by acid production" during exponential growth of L cells. This is in agreement with the findings of Leslie, Fulton and Sinclair (1957) which showed a very close correlation between the amount of

tissue present and the "glucose unaccounted for" during roller tube experiments with HEP 2 carcinoma cells and other malignant and embryonic cell cultures. By assuming that the proportion of this quotient which was assimilated was sufficiently small to ignore for practical purposes, these authors considered that the contribution of excess glycolysis to net ATP production over 72 hours was about 10% in the case of malignant cells and about 20% for embryonic cells. Experiments with 14-C glucose have shown that the glucose assimilated by L cells during exponential growth amounts to 20 - 25% of the glucose unaccounted for by acid production. From the 14-C balance in table 5,6 it was concluded that little, if any, of the remainder is returned to the medium as non-essential amino acids or other metabolites. On the basis that 75% of the glucose unaccounted for by acid production is respired to carbon dioxide, calculations can be made on the approximate rate of production of ATP during growth. The table below contains data from the five control experiments without uncoupler presented in sections A, B and C. Where the quotient for α-keto acids produced is negative, no calculations have been made as in such cases this has been considered in the "glucose unaccounted for" quotient. All quotients are expressed in u moles per mg. protein hour.

	0-24 hours		24-48 hours		48-72 hours		72-96 hours	
	Q	ATP	Q	ATP	Q	ATP	Q	ATP
Lactic acid produced	0.37	0.37	0.30	0.30	0.22	0.22	0.15	0.15
s.d.	0.02		0.04		0.04		0.03	
α-keto acids produced	0.06	0.24	0.0	0.0	-0.02		-0.01	
s.d.	0.02		0.02		0.01		0.01	
Glucose "unaccounted for by fermentation"	0.17	4.85	0.18	5.13	0.17	4.85	0.16	4.52
s.d.	0.01		0.02		0.01		0.02	
Total ATP produced per mg. protein hour		5.46		5.43		5.07		4.67
Proportion of ATP produced by excess glycolysis	11%		6%		4%		3%	
Q O_2 Glucose/protein	17.2		18.2		17.2		16.0	

The results therefore show that for glucose metabolism during growth the contribution of ATP produced by glycolysis falls from about 11% over the first day to 3 - 4% over the third and fourth days. The calculated ATP production from glucose respiration does not differ significantly over the period of growth, although there is a suggestion of a decrease over the last day. The rate of total ATP production is about 15% lower on the fourth day than on the first day and the second day, largely as a result of decreasing glycolysis. No experiments have been done to evaluate the significance of this trend into stationary phase after the fourth day.

The average QO_2 Protein values have been calculated from the values for glucose respired and are shown in the above table. It is of note that they are not significantly different from those obtained in manometric studies with glucose as substrate (graph 19). The quotient for lactate produced during the first day of growth is also of the same order as that noted for cells in manometric conditions at pH 7.4. This similarity is rather surprising in view of the considerable differences in suspension medium, cell: medium ratios, physical conditions and length of time. It is in fact in contrast to the findings of Rucker et al (1963) that the quotients for glucose respired during growth of HeLa and chick embryo cells were considerably lower than for cells in manometric experiments. The points may be made, however, that in Rucker's experiments the cells were set up in conditions to which they were not adapted, that is, medium without serum; considerable necrosis occurred over the 72-hour experimental period and respiration rates calculated from glucose respired were very low. The data obtained for L cell metabolism during growth gives no information on the extent to which components of the medium other than glucose were oxidised. Manometric experiments (Table 33) show that glutamine can be metabolised by L cells, and other amino acids also must be regarded as potential substrates for oxidation. Although Kitos, Sinclair and Waymouth (1962) showed that glutamine was oxidised to carbon dioxide by L cells even when glucose was present, they concluded that its contribution to ATP production was in fact very

small. It seems likely that ATP calculations based on glucose metabolised are not far wrong in the present study at least.

While the finding that the quotient for glucose respired is relatively constant during log growth is in general agreement with that of Leslie's group (Leslie, Fulton and Sinclair, 1957; Sinclair, 1958) and concurs with the observation by Gifford, Robertson and Syverton (1956) that respiration is proportional to cell number in HeLa cell cultures, it appears to be at variance with the experiments of Danes and Paul (1961) on L cells. These latter results have already been briefly referred to (page 124). They show that after subculture of L cells to fresh medium, the respiration rate fell within 48 hours to under 20% of its initial value. After three days the rate started to rise and on the fifth day when the cells had entered stationary phase, the rate was about ten times greater.

There are, however, some important differences between the methods used in Paul's laboratory and those in the present study. In the former case, the initial inoculum was 50,000 stationary phase cells per ml. and the growth medium was not equilibrated against carbon dioxide and air. In contrast, the experimental medium used in the present study was bicarbonate buffered against 5% carbon dioxide in air and added to exponentially growing cells at a concentration of about 200,000 cells per ml. Danes and Paul suggest that the initial fall in respiration is due to leakage of intermediaries from the newly inoculated cells into the medium until an equilibrium between cells and medium is established. In this respect the low cell: medium ratio may be important in delaying this. Furthermore, it seems likely that stationary phase cells have membranes which are less robust than log growing cells and more liable to lose intermediates. Danes and Paul do not claim that their results show that growth rate is completely divorced from rate of ATP production, although, as has been noted, the respiration rate measured by Cartesian Diver technique during the fastest growth phase was only about 10% of that during

stationary phase. Glycolysis may have been considerably faster in the early days of the experiment and it does not seem inconceivable that the respiration rate in the growth medium was rather faster than the measurements in the Diver suggest: as growth was in slanting stationary tubes the concentration of leaking intermediates in the vicinity of the cells might build up within a few hours sufficiently to slow down the net leakage in a manner which would not be observed under short-term Diver experiments.

The experiments with 14-C glucose showed that substantial quantities were incorporated into cellular material. The percentage of glucose used which was incorporated varied from 11 - 12% over the first 48 hours to 13 - 15% over the last 48 hours. The rate of incorporation during exponential growth was, however, remarkably constant at about 1.8 u gm. atoms 14-C per 100 ug. protein synthesised, or approximately 1.0 u gm. atom nitrogen incorporated into protein. Although some turnover of cell material seems likely, incubation of cells containing 14-C in cold medium did not result in any significant loss of the isotope to the medium. Relatively few reports are available on the contribution of glucose C to cellular material during growth. The composition of the medium and particularly the extent to which non-essential amino acids are present might be expected to influence this greatly. The medium used in this case contains many of the non-essential amino acids, and it seems therefore unlikely that extensive amino acid synthesis should take place. The most likely components to acquire glucose C are the nucleic acids, nucleotides, lipids and structural carbohydrates. Crocket and Leslie (1963) have examined this to some extent for HLM cells in WB 752/1 plus 4% serum. Their results show that between 15 - 25% of the 14-C assimilated was found in protein, and the remainder in other components. Their data indicates that in the region of 2.5 ug. atom C were incorporated for every 100 ug. protein synthesised. Mohberg and Johnson's (1963) results on amino acid utilisation by L cells in MB 752/1 indicate that about 2-3 ug. atoms C from glucose were incorporated for every ug. atom nitrogen assimilated

into protein. Insufficient data is available for precise calculation. The results presented here, therefore, are in reasonably good agreement with others, and underline the importance of glucose as a substrate for synthetic reactions.

On the basis that 75% of the glucose unaccounted for by acid production is respired, it is possible to make some calculations relating protein synthesis approximately to the amount of ATP available to the cell, assuming glucose to be the major energy source. The data from the first 48 hours of the five control experiments in sections A, B and C shows that 36.2 s.d. 2.8 u moles ATP were produced (and presumably utilised) during the synthesis of 100 ug. protein. It must be emphasised that this is the ATP yield from glucose metabolism only and should therefore be taken as a minimum. It can be calculated from the amino acid analysis carried out by Mohberg and Johnson (1963) that the average amino acid of L cell protein contains approximately 1.3 ug. atoms nitrogen per u mole. On this basis the synthesis of 47 moles ATP by an exponentially growing system is related to cellular activities, an index of which is the formation of one peptide bond. This is difficult to compare meaningfully with results from other laboratories since in most cases the glucose assimilated fraction has not been determined, making calculation of the actual glucose respired in error by this amount. Observing that his results were incorrect in this respect, Sinclair (1958) calculated that in malignant cell lines H.Ep.1, H.E.p.2 and HLM growing in Connaught's medium supplemented with serum, approximately 52 moles ATP were formed per peptide bond. In the case of embryonic lung and kidney cells, 57 moles ATP were synthesised per bond. Sinclair estimated from the data of Gifford, Robertson and Syverton (1957) on HeLa cells that the relevant figure in this case was 56 moles ATP. This calculation was, however, based on respiration rates and therefore the energy is that derived from glucose and any other substrate which the cell may be oxidising. Allowing for the fact that Sinclair's

results are high as some of the "glucose unaccounted for" was assimilated and that Gifford et al's figure probably includes some ATP produced from substrates other than glucose, these figures covering six different cell strains are in close agreement with the results for L cells.

Consideration of other cases, however, implies that there is no universal relationship between the production of ATP and cellular protein synthesis. For example, the data of Westfall et al (1956) on the growth of mouse cells, indicates that 140 moles ATP were formed from glucose for every gm. atom nitrogen incorporated. Conversely analyses of the data provided by Crockett and Leslie (1963) on the growth of HLM cells in WB 752/1 plus serum, and Connaught's 858 plus serum and embryo extract show that approximately 20 - 30 moles ATP were synthesised from 14-C glucose per g.atom nitrogen incorporated, assuming that 5% of the total acids produced were α-keto acids.

(ii) THE EFFECTS OF 2:4-DNP ON GROWTH AND METABOLISM UNDER ANAEROBIC CONDITIONS

Several reports indicate that certain mammalian cell types can grow equally well under aerobic and anaerobic conditions. This seems to be especially so for embryonic tissues, such as chick intestine and lung (Jones and Bonting, 1956) and chick fibroblasts (Pomerat and Willmer, 1939). Other reports indicate that reduced but constant growth rates can be maintained under anoxia. Dales (1960) for instance, has concluded that under anaerobic conditions, cells grow at rates approximately proportional to the theoretical ATP yield, and found that L cells could grow apparently indefinitely with mean generation times about three times longer than under aerobic conditions.

The present findings on L cells (Graph 11, Tables 13 and 14) differ from those of Dale on two points - the first in that growth could not be maintained for longer than 48 hours, and the second that the glucose utilised under anaerobic conditions could substantially be accounted for by acid production. With respect to growth under anoxia, there can be little doubt that this depends to a large extent on the nutrient medium used. Dales used CMRL 1066 supplemented with 20% horse serum. This medium contains the deoxyribosides of adenine, guanine and cytidine, and is obviously richer than MB 752/1 by many of the components present in the serum also. Indeed it is difficult to see how cells could synthesise nucleic acids from the components of MB 752/1 other than through oxidative pathways. (This question is considered in a later section.) It is proposed therefore that one reason for the observed rapid cessation of growth, and subsequent necrosis, is depletion of intracellular components which are not available from the medium and require oxidative pathways for synthesis. As already pointed out, despite the careful gassing procedure used, the possibility of anaerobiosis being incomplete at least initially cannot be entirely discounted. It is of relevance that,

although synthesis over the first 48 hours approximates to 28% of that of aerobic controls, the ATP production under anoxia was no more than about 15% of aerobic rates. To assume complete anaerobiosis, therefore, is to assume that ATP production and cell synthesis are very loosely coupled processes.

As 2:4-DNP does not uncouple substrate level phosphorylations, an intriguing aspect of the effects of 2:4-DNP on anaerobic cells is the extent to which the uncoupler accelerates necrosis. This finding is entirely supported by the results of manometric experiments discussed elsewhere. In the light of misgivings on the completeness of respiratory inhibition in the growth experiments, it may be argued that the stimulation of "anaerobic glycolysis" by 2:4-DNP is primarily due to the uncoupling of any respiratory chain phosphorylations which may be occurring. However, the observation of an identical phenomenon under careful manometric conditions clearly suggests that in the case of growth too, stimulation of ATPase activity is the most plausible explanation for both stimulation of anaerobic glycolysis and acceleration of necrosis.

(iii) THE EFFECTS OF 2:4-DNP ON GROWTH AND METABOLISM

The effects of 2:4-DNP on growth and metabolism are in general, very similar to those observed and commented upon in manometric studies. Addition of the uncoupler at concentrations between 0.05mM and 0.15mM results in increased rates of acid production and utilisation of glucose unaccounted for by glycolysis, and decreased growth rates. Investigation of cell growth and metabolism over four days has shown that patterns of metabolism imposed by 2:4-DNP change with time in a predictable and characteristic manner. Uncoupling, in fact, imposes conditions on the cell which progressively lead to a decrease in pH with a concomitant increase in the proportion of the undissociated 2:4-DNP molecules in the medium. As the activity of the uncoupler apparently increases with the undissociated molecule, such a system is autocatalytic and inexorably leads to necrosis and death. Cells treated with uncoupler appear in fact to lose all ability to regulate their environment relative to pH. This is shown in graphs 1 - 3. Depending upon the concentration of uncoupler, decreasing pH is accompanied by increasing rates of acid production and utilisation of "glucose unaccounted for" (0.05mM 2:4-DNP) increasing acid production and decreasing "glucose unaccounted for" (0.10mM) and decreasing acid production and decreasing "glucose unaccounted for " (0.15mM). In each case the cells, although perhaps able to grow under the initial conditions imposed, produce in an autocatalytic manner the conditions for necrosis.

Cells under growth conditions respond in a different quantitative manner to 2:4-DNP than cells under manometric conditions. The table below shows the quotients for lactate produced under growth conditions at pH 7.4 and manometric conditions at the same pH.

u moles lactate produced per mg. protein hour						
2:4-DNP	-	0.05	0.10	0.125	0.15	source of figures
GROWTH	0.38	0.61	0.82	1.24	1.46	Graphs 2,9, Tables 4-6,11-14 5-7 quotients for first 24 hours
s.d.	0.02	0.04	0.10	-	0.09	
MANOMETRIC	0.40	1.23	1.80		2.23	Graph 20
s.d.	0.02	0.05	0.06		0.08	

If QO_2 values are calculated for growth experiments on the basis that in the absence of uncoupler, 75%, and in the presence of 0.05, 0.10, 0.125 and 0.15 mM 2:4-DNP, 85%, 90%, 95% and 95% respectively of the quotient "glucose unaccounted for by acid production" is respired, the table below can be constructed. (N.B.: only in the case of no 2:4-DNP and 0.10mM 2:4-DNP have the values for 14-C respiration been established experimentally (Table 5-7). The values for the others have been assumed from these and are therefore approximate. The margin of error involved in such assumptions however makes no essential difference to the general principle which comparison of the results from growth and manometric experiments reveals.

ultrs O_2 respired per mg. protein hour						
2:4-DNP	-	0.05	0.10	0.125	0.15	source of figures
GROWTH	17.1	25.2	36.2	36.8	30.5	Graphs 2,9, Tables 4-6,11-14 5-7 quotients for first 24 hours
s.d.	0.5	0.6	1.0	-	1.0	
MANOMETRIC	18.1	40.0	38.2		27.3	Graph 19
s.d.	0.9	1.4	1.2		2.2	

Quotients from growth experiments are for oxidation of glucose (see page 166).

A significant feature of both quotients is that 2:4-DNP appears to produce its effects at lower concentrations in the case of the manometric experiments. The maximum rate of lactate production inducible by uncoupler has not been measured for growth experiments due to the rapid necrosis of cells at high uncoupler concentrations, but it is clear that at concentrations of 2:4-DNP up to 0.15mM, the rates are much lower than those induced by similar concentrations

under manometric conditions. Whereas the maximum QO_2 for manometric experiments is induced by about 0.075mM uncoupler (graph 19), the maximum QO_2 (Glucose) for growth experiments requires about 0.10 - 0.125mM uncoupler. Such features suggest that the uncoupling activity of dinitrophenol is lower in growth experiments than in manometric experiments, although the pH was similar in both groups. Further consideration of the response of metabolism and growth to the uncoupler indicates that this is so.

* * * *

Respiratory chain phosphorylations do not appear to be completely uncoupled by 0.05mM 2:4-DNP. Although the rates of glycolysis and respiration (indicated by the "glucose unaccounted for" quotient) are greater than those for the control cultures, they are considerably lower than those induced by 0.10mM uncoupler. Furthermore, calculation of substrate level phosphorylations relative to growth indicates that the ATP produced from these along would be unlikely to support the observed growth. For example, consideration of the data on growth in the presence of 0.05mM uncoupler shown in graphs 1 and 2, reveals that over the first 48 hours before the pH had fallen below 7.0, the rate of ATP synthesis was 17 a.d. 0.8 moles ATP per peptide bond formed. This compares with 47.1 s.d. 3.6 moles ATP per peptide bond in the absence of uncoupler. It is suggested then that a growth rate such as that in the presence of 0.05mM 2:4-DNP is incompatible with complete uncoupling of respiratory chain phosphorylations.

The most reasonable interpretation of growth and metabolism at 0.10mM 2:4-DNP is that uncoupling is substantially but not quite complete even at this concentration. The table on page 171 shows that, subject to the assumptions made then, glucose respiration is on average twice that of the controls and is nearing its maximum 2:4-DNP induced rate. This has been discussed

page 146) as one of the criteria for complete uncoupling. Although increased glycolysis occurs at 2:4-DNP concentrations greater than 0.10mM, it has already been suggested that this might occur even when cells are completely uncoupled due to increased ATPase activity. Calculation of the ATP production from substrate level phosphorylations relative to growth shows that an average of 30.3 s.d. 2.4 moles ATP are produced per peptide bond formed over the first 48 hours, for the five experiments where the pH is uncontrolled but has not fallen below 7.0 during this time. In pH controlled experiments (graphs 4 and 6) 27.8 a.d. 1.3 ATP are produced per peptide bond formed over the 96 hours. These figures taken together, are significantly lower than the 47.1 s.d. 3.6 for the controls ($P < 0.01$). A further point which must be kept in mind is that the effective ATP production may well be less than the theoretical due to stimulated ATP ase activity. Furthermore, if substrates other than glucose contribute to ATP production by entering directly into the TCA cycle, this would increase the ATP moles per peptide bond in the control cultures, while making little difference to the calculated substrate level ATP per peptide bond in the 2:4-DNP treated cells. Perhaps the best evidence for believing that complete uncoupling is not achieved by 0.10mM uncoupler under these conditions is the finding presented later that the limited growth which takes place with pyruvate as substrate instead of glucose is not completely suppressed by this concentration of uncoupler. The weight of evidence therefore seems to be against the idea that only substrate level phosphorylations occur at 0.1 mM 2:4-DNP under these circumstances. In this respect 0.1mM 2:4-DNP differs in its effects on cells in bicarbonate buffered growth medium and in phosphate buffered balanced salt medium, as in the latter case it was concluded that uncoupling was probably complete. This concentration of uncoupler is however the greatest concentration in which growth can be consistently sustained for any length of time, and that only by maintaining the pH at 7.1 - 7.4.

Table 4 shows that at 0.125mM 2:4-DNP no significant growth occurs although the cells maintain themselves for some time with no marked reduction in metabolic quotients if the pH is maintained about the physiological value. The cells are therefore very sensitive to relatively small differences in the quantity of uncoupler around this concentration. From the data in Table 4 it may be calculated that the mean rate of ATP production by substrate level phosphorylation over 4 days in 0.125mM 2:4-DNP is 2.5 s.d. 0.1 u moles per mg. protein hour. This compares with 5.2 s.d. 0.4 u moles in the case of the controls, which were growing exponentially over the four days. This shows that at the higher concentrations of uncoupler, theoretical ATP production from substrate level phosphorylations is quite unrelated to the net protein synthesis. In view of the finding of Dales (1960) that growth of L cells under anaerobic conditions was directly related to the rate of ATP production, and other evidence discussed in the preceding pages to indicate that ATP production and growth were, in general, closely related phenomena, the most likely explanation of the complete inhibition of growth by 0.125mM uncoupler is that ATPase activity is very high at this concentration and competes successfully with metabolic and synthetic pathways for ATP. It cannot be ruled out that failure to grow in this concentration of uncoupler is due to an inhibitory effect of the phenol on a specific enzyme system important for synthetic reactions. At present, however, no evidence exists that this can occur with concentrations of this order.

In contrast to the ability of cells to maintain themselves for some time in the presence of 0.125mM uncoupler, necrosis is inevitable in 0.15mM even when the pH is maintained at physiological level. Necrosis appears to be unrelated to theoretical substrate level phosphorylations. Although as Graph 2 shows, the "glucose unaccounted for" quotient is depressed from the maximum 2:4-DNP induced rate, the high rate of glycolysis over the first 24 hours indicates a mean rate of ATP production from substrate level phosphorylations

of 2.5 a.d. 0.2 u moles per mg. protein hour. This is not significantly different from that at 0.125mM uncoupler and is almost 50% of the rate of control cultures growing exponentially. It might be postulated that hydrolysis of ATP by dinitrophenol stimulated ATPase activity is even greater at 0.15mM concentration, possibly reducing the ATP available for cellular activities to a level which is unable to maintain organisation for long. Further studies are required to assess the effects of the phenol on other enzyme systems, however, before necrosis can be definitely attributed to insufficient ATP.

Despite the general similarities of the action of 2:4-DNP under manometric and growth conditions, certain anomalies have appeared. At lower concentrations its effects are greater under manometric conditions than during growth. This may well by a consequence of the buffers used: as stated elsewhere the bulk pH of cell medium does not necessarily indicate the pH at the cell surface and it may be that this is rather less well controlled in the case of the phosphate buffered manometric medium (page122). A consequence of this could be a difference in the effective concentrations of undissociated molecules of uncoupler at the cell surface in the different media.

At higher concentrations of uncoupler, its effects on cell maintenance and growth appear to be more severe than indicated by the manometric studies. For example, a feature of manometric studies was the steady state system established by 0.20mM 2:4-DNP. Growth studies show that this cannot exist for more than a few hours, thereby illustrating the importance of supporting observations on the primary effects of an antimetabolite with information on its cumulative effects.

Despite the abnormal metabolism, growth in 0.1mM 2:4-DNP appears to be stable and balanced over long periods if the pH is maintained above about 7.1. Over ten weeks exponential growth, no significant changes were found in the proportions of gross cellular components such as ribose, deoxyadenosine equivalents and protein. Enzyme assays, however, showed that uncoupling

induces changes of a less obvious nature. The increases in LDH and aldolase activities have several intriguing features. Firstly, although respiration and glycolysis proceeded faster in 0.1mM treated cells than in 0.05mM treated cells, the increases were only clearly significant at the lower concentration. Stimulation of enzyme activity was therefore more closely related to rate of protein synthesis than to stimulation of glycolysis. A further point is that the noted increases did not confer any obvious advantages on cells growing in 2:4-DNP. Clearly the maximum rate of glycolysis induced by 0.05mM 2:4-DNP is not limited by enzymes of the EMP, as considerably faster rates can be induced by higher concentrations of uncoupler. Furthermore, the rapidity with which the rates of glycolysis change on uncoupling in the case of manometric experiments makes it clear that increased enzyme synthesis is not essential for the attainment of these rates which may be as high as four times the rate induced by 0.05mM uncoupler in growth experiments. It seems likely therefore that the increased levels of LDH and aldolase develop in response to stress upon the systems although these are far from saturated. Although the stress upon the EMP is much greater during growth in 0.10mM uncoupler, the production of ATP is much less and possibly limits enzyme synthesis. Paul (1959) examined the concentrations of enzymes including esterase, acid phosphatase, alkaline phosphatase and glucose-6-phosphatase in L cells. He concluded that enzyme concentrations in cultured cells do not change readily, but showed that glutamine transferase activity could be increased by a lowering of glutamine concentration. Since then it has been shown by Adebonojo, Bensch and King (1960) that L cells incubated under nitrogen/CO2 for 16 hours daily increase their LDH and aldolase activity and lose a substantial proportion of cytochrome oxidase activity. It is of interest that the results on the effects of uncoupling resemble the effects of anaerobiosis on LDH and aldolase activities. As already remarked,

uncoupling and anoxia induce increases in glycolytic rate and decreases in synthesis, and are similar in this respect. This suggests that it might be of interest to examine the enzyme concentrations of cells maintaining themselves at very high pH with a correspondingly high rate of glycolysis, relative to the effects of uncoupling and anoxia.

(iv) THE EFFECTS OF SODIUM SALICYLATE ON GROWTH AND METABOLISM

The concentrations of salicylate required to produce effects on growth and metabolism were much higher than those of 2:4-DNP. For example, 4mM salicylate was required to produce an inhibitory effect on growth roughly comparable to that of 0.05mM 2:4-DNP, and marked necrosis was brought about by 10mM salicylate compared to 0.15mM 2:4-DNP. At pH 7.4 therefore, salicylate was approximately 70 - 80 times less effective than 2:4-DNP, on a molar basis. Furthermore, although there was a general similarity between the effects of the two uncouplers on growth and metabolism, the maximum effects induced by salicylate on acid production and glucose "unaccounted for by acid production" were much less marked than by 2:4-DNP. For example, the maximum increase induced by salicylate in the rate of lactate production over the first 24 hours was about 20%. This compares with increases of up to 350% induced by 2:4-DNP. The greatest increase in the quotient for "glucose unaccounted for by fermentation" was 25% by salicylate compared with about 70% induced by 2:4-DNP. In these respects the results of growth experiments confirm the findings in the short-term manometric experiments already discussed.

On page 157 it was concluded that in the case of sodium salicylate, the anticipated increases in respiration and glycolysis on uncoupling were in large measure prevented by an inhibitory effect of the uncoupler on enzymes involved in these processes. Under such conditions, where substrate level phosphorylations are not greatly increased, complete uncoupling would decrease the rate of production of ATP from glucose by about 85%. Taking stimulation of ATPase activity into account, it would appear virtually impossible for cells completely uncoupled by salicylate to maintain themselves, far less grow. Graph 13 shows that limited growth occurs over 4 days in 4mM salicylate, while at 10mM, necrosis occurs. This suggests that complete uncoupling

probably occurs at some concentration between 4mM and 10mM salicylate, and is in good agreement with the conclusion reached from the manometric experiment that under these conditions complete uncoupling occurred at about 7mM.

The toxic effects of salicylate cannot be entirely attributed to interference with ATP metabolism through uncoupling and inhibition of associated oxidative and glycolytic processes, or even the effects of these on loss of potassium, for example (Manchester, Randle and Smith, 1958; Hicklin, 1958). Substantial evidence exists that salicylates also inhibit a variety of enzymes involved in synthetic pathways. Bollet (1961) has shown that the drug reduces the synthesis of glucosamine-6-phosphate from fructose-6-phosphate and glutamine in homogenates of rat liver and connective tissue, and suggests a direct inhibition of transamidase activity. Huggins, Smith and Moses (1961) noted that salicylate differed from 2:4-DNP in that it decreased incorporation of $^{3}14$-C pyruvate into glutamic acid in rat kidney homogenates, and subsequently showed that it inhibited rat serum glutamic-pyruvic transamidase in vitro. Smith (1963) has stressed the importance of further investigation into these aspects of salicylate action with respect to the production of peptide substances such as kinins which may be implicated in the maintenance of inflammatory reactions. The basis of the anti-inflammatory action of salicylate is far from understood (Marks, Smith and Cunliffe, 1961; Spector and Willoughby, 1963) and the information from L cells makes little contribution to the problem. It is of some interest, however, that the normally effective therapeutic concentrations in the blood - 2mM - 4mM (Ansell, 1963) lie within the concentration range which produces significant effects on L cell growth and metabolism. Despite the specialisation of this cell type to growth in tissue culture, its ability to grow in chemically defined medium indicates that investigations along the lines suggested by Smith (1963) might profitably be pursued.

SECTION 2

EXPERIMENTS WITH PYRUVATE AND LACTATE AS SUBSTRATES

E X P E R I M E N T A L

Tables and Graphs are presented at the conclusion of each sub-section

MANOMETRIC EXPERIMENTS

(i) Respiratory Rates with various substrates

The results (Table 33) of manometric experiments with various substrates at 10mM concentration (succinate 25mM) show that the highest respiratory rates were obtained with pyruvate, lactate, glutamine and glucose. The rates with these substrates were on average, respectively 1.93, 1.63, 1.48 and 1.46 times the endogenous rates. Succinate and α-ketoglutarate were the only other substrates tested which consistently stimulated respiration, and the rates induced by these substrates were only 1.16 and 1.23 times respectively the endogenous rates. Addition of citrate, ribose and alcohol did not produce significant increases over the endogenous rates. In all cases the rates were constant with time for two hours or more, except with alcohol when the rate fell by about 30% over the two hours.

(ii) Optimum Pyruvate concentration for respiration

As pyruvate was notably successful as an oxidisable substrate, further investigations were made into its metabolism by L cells. Graph 27 shows the results of an experiment to determine the optimum pyruvate concentration for respiration. The QO_2 values plotted were constant for 20, 35, 50 and 120 minutes respectively at initial pyruvate concentrations of 0.1, 0.25, 0.5 and over 1.0mM, and were determined over these periods of time. It was concluded that respiration proceeds at maximum rate at external pyruvate concentrations of 0.5mM to 25.0mM.

(iii) Effects of 2:4-DNP on the metabolism of Pyruvate and Lactate

The respiration rates of pyruvate and lactate were considerably increased by 2:4-DNP. Graph 28 contains data from four experiments with pyruvate as substrate and two with lactate, respiration being measured over one hour in

each case. Flasks with glucose as substrate were included in these experiments from time to time, but the data for glucose shown in this Graph is that from Graph 19 (pH 7.4) and has been included for comparison. The maximum respiratory rates with pyruvate and lactate were induced by 0.1mM 2:4-DNP. This is rather more uncoupler than is required to induce the maximum rate with glucose as substrate (0.075mM). Although the respiration rate with pyruvate was significantly higher than that with glucose, the maximum 2:4-DNP stimulated rates with these substrates were approximately the same, the QO_2 values being between 42 and 45. The maximum 2:4-DNP induced rate with lactate as substrate was on average about 20% lower than that for pyruvate and glucose. Concentrations of uncoupler in excess of 0.1mM (pyruvate and lactate) progressively inhibited respiration in a manner already observed and commented upon for glucose.

Table 34 contains results from two experiments with pyruvate as substrate in which analysis of the medium was undertaken. During the experiments, which lasted 1 hour, the respiration rates with pyruvate present remained constant with time, even at 0.5mM 2:4-DNP, and it was assumed that necrosis was not significant. The quantity of pyruvate used by the cells increased with uncoupler concentration to about 0.10mM 2:4-DNP. At higher concentrations with increasing inhibition of respiration and lactate production, the uptake of pyruvate from the medium was reduced and at 0.5mM was on average 27% lower than that of controls without 2:4-DNP.

In the absence of 2:4-DNP 33-36% of the pyruvate used was accounted for by the production of lactate into the medium. With increasing concentrations of uncoupler, the lactate production decreased and at 0.1mM 2:4-DNP (the concentration inducing maximum respiration rate) was only 50% of that of controls without the uncoupler. Higher concentrations of uncoupler produced no further inhibitory effect on lactate production, and it is of interest that

at such concentrations, which very significantly reduced respiration rate, the percentage of total pyruvate used converted to lactate increased.

The problems of calculating the quantities of substrate oxidised and assimilated during experiments have been mentioned elsewhere. In the absence of direct measurements of 14-C pyruvate metabolism, it is not possible to assess precisely either the extent to which pyruvate depresses endogenous respiration or the measure in which it is assimilated. The figures obtained on the assumption that pyruvate does not depress endogenous respiration show that pyruvate used which is unaccounted for by lactic acid production and oxidation, decreases with increasing 2:4-DNP. At 0.5mM for example, this was less than 50% of that for the controls without uncoupler. If, however, exogenous pyruvate depressed endogenous respiration as glucose appears to (page 104) the figures for pyruvate oxidised are too low, and those for pyruvate unaccounted for too high. Since it seems reasonable to suppose that this occurs to some extent at least, the inhibitory effects of uncoupling on pyruvate assimilation are likely to be rather greater than Table 34 suggests.

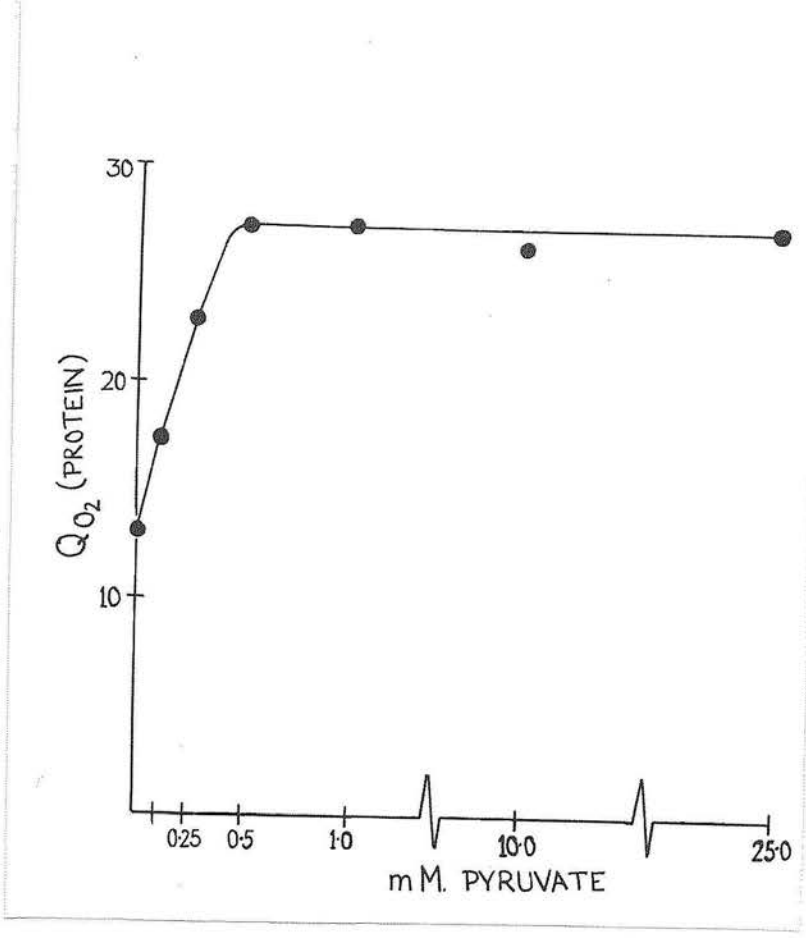
(iv) The Effects of Glucose on Pyruvate Metabolism

The results set forth in Table 35 show that glucose (10mM) added to cells metabolising pyruvate (10mM) decreased the respiration rate and increased the rate of lactate production. The decrease in respiration rate was however small (5 - 14%) and the new rate was still about 20% greater than the rate in glucose alone. The effects of glucose on lactate production were greater than those on respiration rate, and an increase of 25 - 40% was observed. The rate of lactate production by cells in pyruvate plus glucose was however always less than that in glucose alone.

TABLE 33

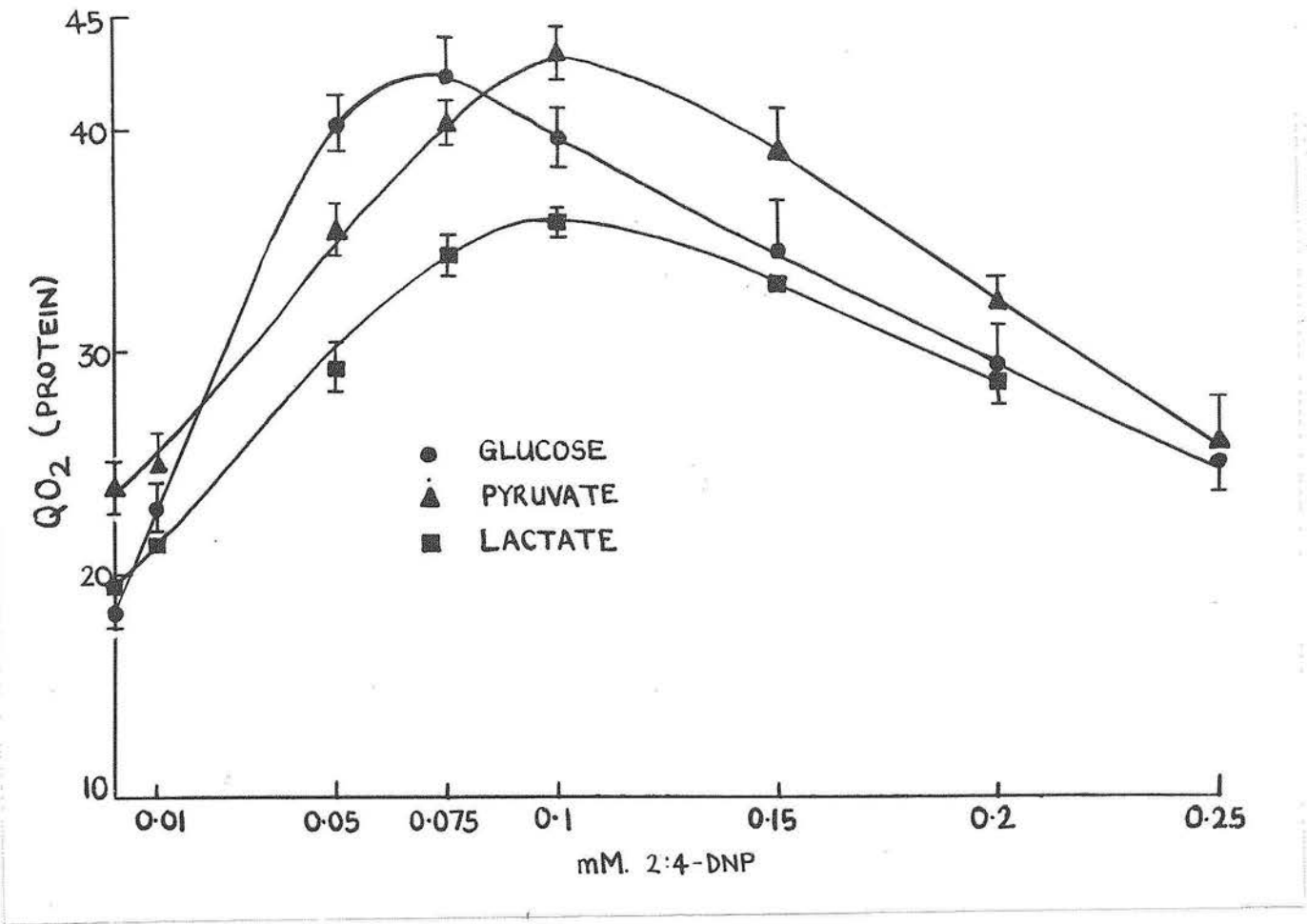
RESPIRATION RATES WITH VARIOUS SUBSTRATES

Substrate	Exp. 1 $Q0_2$	2 $Q0_2$	3 $Q0_2$	4 $Q0_2$	$\Sigma \frac{Q0_2 (Substrate)}{Q0_2 (endog)}$	s.d.
Endogenous	13.2	12.1	12.3	13.9	1.00	
Glucose	18.1	18.9	17.2	20.9	1.46	0.08
Pyruvate	25.2	22.8	24.8	-	1.93	0.06
Lactate	19.7	21.6	19.8	-	1.63	0.10
Glutamine	20.2	19.0	-	18.9	1.48	0.08
α Ketoglutarate	14.8	17.3	14.0	-	1.23	0.13
Succinate	-	13.9	14.2	-	1.16	0.00
Citrate	11.1	13.0	12.3	-	0.96	0.09
Alcohol	- -	- -	- -	12.8 13.2	0.94	0.02
Ribose	-	12.5	12.3	-	1.01	0.02



GRAPH 27

RESPIRATION RATES WITH PYRUVATE



GRAPH 28

EFFECTS OF 2:4-DNP ON RESPIRATION OF PYRUVATE (4 experiments) LACTATE (2 experiments) and GLUCOSE (Graph 19)
Standard Deviations shown.

TABLE 34

PYRUVATE METABOLISM OVER ONE HOUR IN KREBS RINGER PHOSPHATE AT pH 7.4

2:4 Dinitrophenol	-	0.05	0.10	0.25	0.50	mM
Experiment 1: 8.5 m.g.protein						
Respiration (Endogenous)	109	117	109	80	77	u ltrs O_2
Respiration (Pyruvate 8mM)	200	303	368	269	195	u ltrs O_2
QO_2 (Pyruvate)	23.6	35.7	43.4	31.7	23.0	u ltrs O_2
Pyruvate utilised	5.82	7.20	7.00	4.91	4.35	u moles
Lactic acid produced	1.92	2.15	1.08	1.03	1.22	u moles
Pyruvate oxidised	1.62	3.32	4.60	3.40	2.11	u moles
Pyruvate unaccounted for	2.28	1.73	1.32	0.48	1.02	u moles
% converted to lactic acid	33	29	15	21	28	
% unaccounted for	39	24	19	10	24	
Experiment 2: 10.3 mg protein						
Respiration (Endogenous)	140	150	158	110	98	u ltrs O_2
Respiration (Pyruvate 8mM)	225	379	460	325	222	u ltrs O_2
QO_2 (Pyruvate)	21.7	36.8	44.5	31.5	21.5	u ltrs O_2
Pyruvate utilised	5.54	7.23	7.42	6.15	4.00	u moles
Lactic acid produced	1.97	1.49	0.96	1.10	1.02	u moles
Pyruvate oxidised	1.52	4.12	5.40	3.82	2.21	u moles
Pyruvate unaccounted for	2.05	1.62	1.06	1.23	0.77	u moles
% converted to lactic acid	36	20	13	18	25	
% unaccounted for	37	22	14	20	19	

TABLE 35

THE EFFECTS OF GLUCOSE ON THE METABOLISM OF PYRUVATE AT pH 7.4

	Exp.1			Exp.2			Exp.3		
	n.	QO_2	$Q_{L.A.}$	n.	QO_2	$Q_{L.A.}$	n.	QO_2	$Q_{L.A.}$
Pyruvate (10mM)	2	24.0 +-1.2	0.25 +- 0.03	2	24.6 +-0.6	0.18 +-0.04	2	24.8 +-0.6	0.21 +-0.02
Pyruvate (10mM) + Glucose (10mM)	2	22.7 +-0.5	0.35 +-0.02	2	21.2 +-1.0	0.27 +-0.03	2	21.8 +-0.5	0.28 +-0.00
Glucose (10mM)	2	18.3 +-0.4	0.39 +-0.02	2	18.8 +- 0.3	0.41 +-0.04	1	17.7	0.46

n = number of samples

ROLLER BOTTLE EXPERIMENTS

Roller bottle techniques (page44) provide a means of achieving a high cell:medium ratio under physiological and growth conditions. These were resorted to primarily to obtain reliable information on the utilisation and production of acids in growth medium over relatively short periods of time. Thick monolayers of cells were used and possibly as a result of this the increase in total protein was never more than about 15% and not significantly different for any of the substrates used over the 24 hours of most experiments. The protein content per culture was therefore measured only at the end of experiments and used merely to check that the quantities of tissue per culture were approximately the same.

Graphs 29 a - c show the results of a typical experiment comparing the rates of utilisation of substrate where glucose (10mM), pyruvate (10mM) and glucose (10mM) + pyruvate (10mM) were supplied to the cells.

With glucose as the sole carbohydrate substrate this was utilised at a relatively constant rate for 24 hours (Graph 29a). No α-keto acids were produced in to the medium. Lactate was produced in significant quantities during the first few hours only and even then accounted for no more than 10% of the glucose utilised. This pattern of metabolism is therefore very similar to that noted for monolayer cultures approaching stationary phase (page79).

The metabolism of pyruvate (Graph 29b) was strikingly similar to that observed in manometric experiments. It was rapidly removed from the medium at a relatively constant rate until the concentration was about 0.5mM when a sharp decrease was noted. Lactate was produced into the medium at a constant rate until the rate of pyruvate utilisation decreased. The cells then utilised lactate from the medium but at a much lower rate than that observed for pyruvate. During the phase when the rates of pyruvate utilisation and lactate production were constant about 25-30% of the pyruvate utilised could be accounted for by

lactate production.

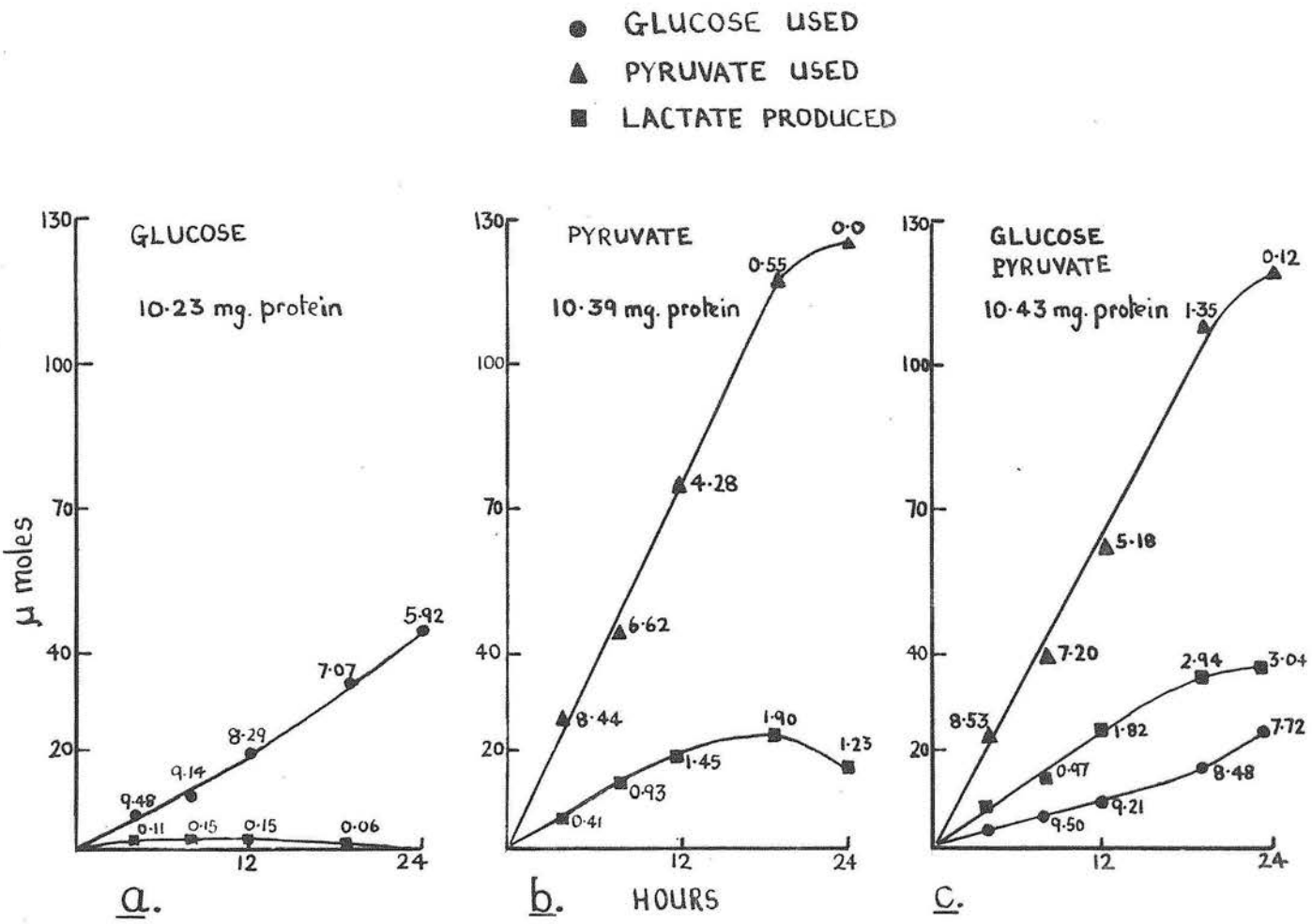
Graph 29c shows that pyruvate depressed the rate of glucose utilisation by over 50%. The presence of glucose depressed the rate of pyruvate utilisation by only 15%. Lactate production was 30% greater than the combined rates of the cultures with pyruvate only and glucose only. The suppression of glucose utilisation by pyruvate was investigated further to establish the minimum pyruvate concentration which induced this effect. Graph 30 shows that as the concentration of pyruvate in the medium decreased below 0.5mM the inhibition of glucose utilisation was progressively released.

Table 36 presents the results of several experiments similar to that shown in Graphs 29 a-c. The figures presented indicate the rates of substrate utilisation and acid production over the first 12 hours of each experiment, and substantiate the observations already made on the metabolism of pyruvate and glucose by roller bottle culture. The theoretical ATP production calculated on the assumption that all the substrate used unaccounted for by lactate production is oxidised is 12-18% greater in the case of cells in pyruvate-containing medium than cells in glucose-containing medium. The actual difference in the rate of ATP production must be greater than this, as certainly a proportion of the substrate used unaccounted for by lactate production must be assimilated, and later experiments indicate that if anything, this proportion would be greater with glucose as substrate than with pyruvate. A greater rate of ATP production in pyruvate media is in agreement with the observations from manometric experiments that the respiration rate with pyruvate as substrate is consistently higher than that with glucose.

Graphs 31 a-c show the results of a typical experiment on the utilisation of pyruvate (10mM) and lactate (10mM)by cells in nutrient media. The initial rate of lactate utilisation was about 20% less than that for pyruvate and decreased with time. When both substrates were added together, the pyruvate was utilised

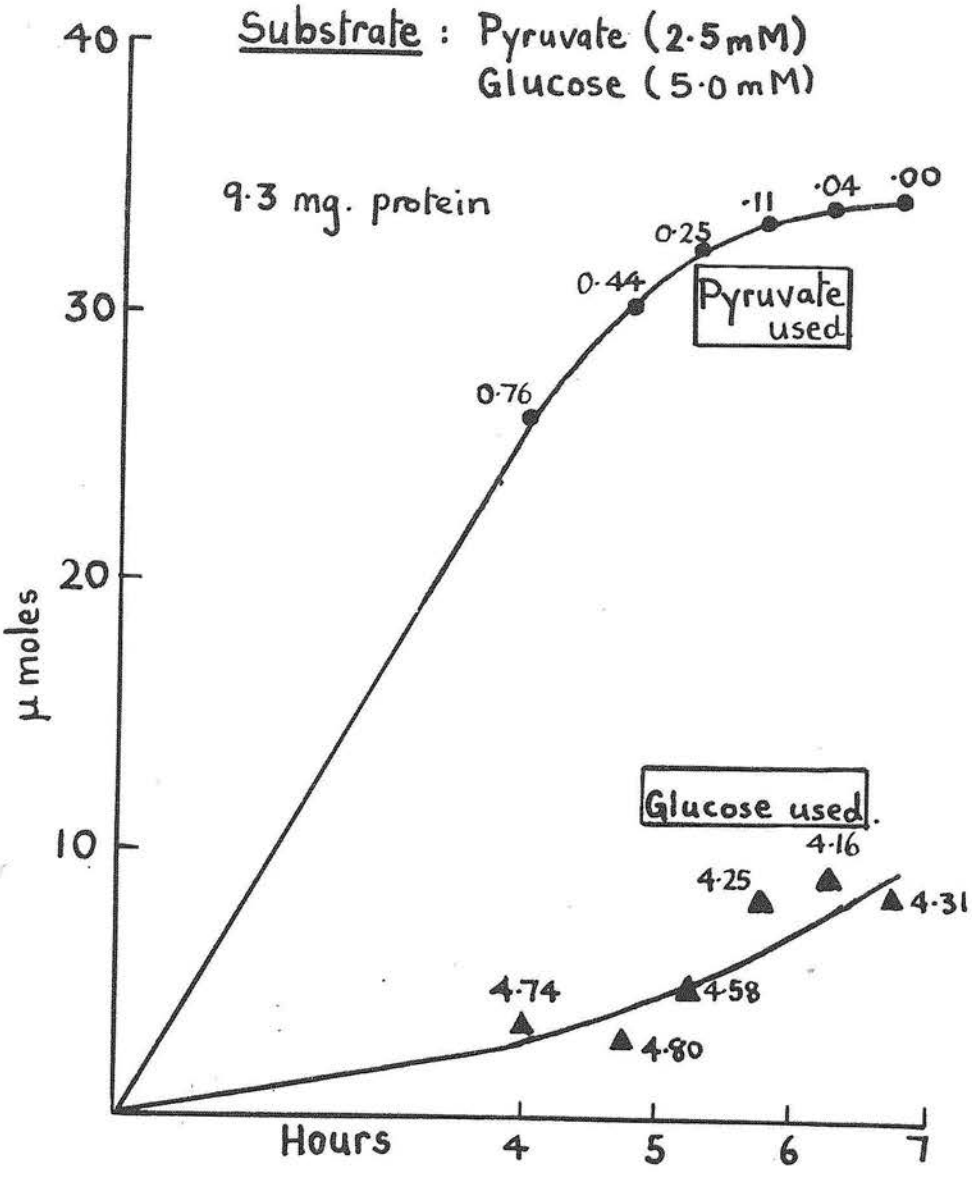
almost completely with a net increase in the exogenous lactate before this too started to decrease.

Roller bottle experiments show therefore that pyruvate is metabolised in preference to exogenous lactate which is, however, slowly metabolised in the absence of the former.



GRAPH 29 a - c

METABOLISM IN MEDIA CONTAINING GLUCOSE, PYRUVATE and GLUCOSE plus PYRUVATE. Figures denote mM. substrate in media.



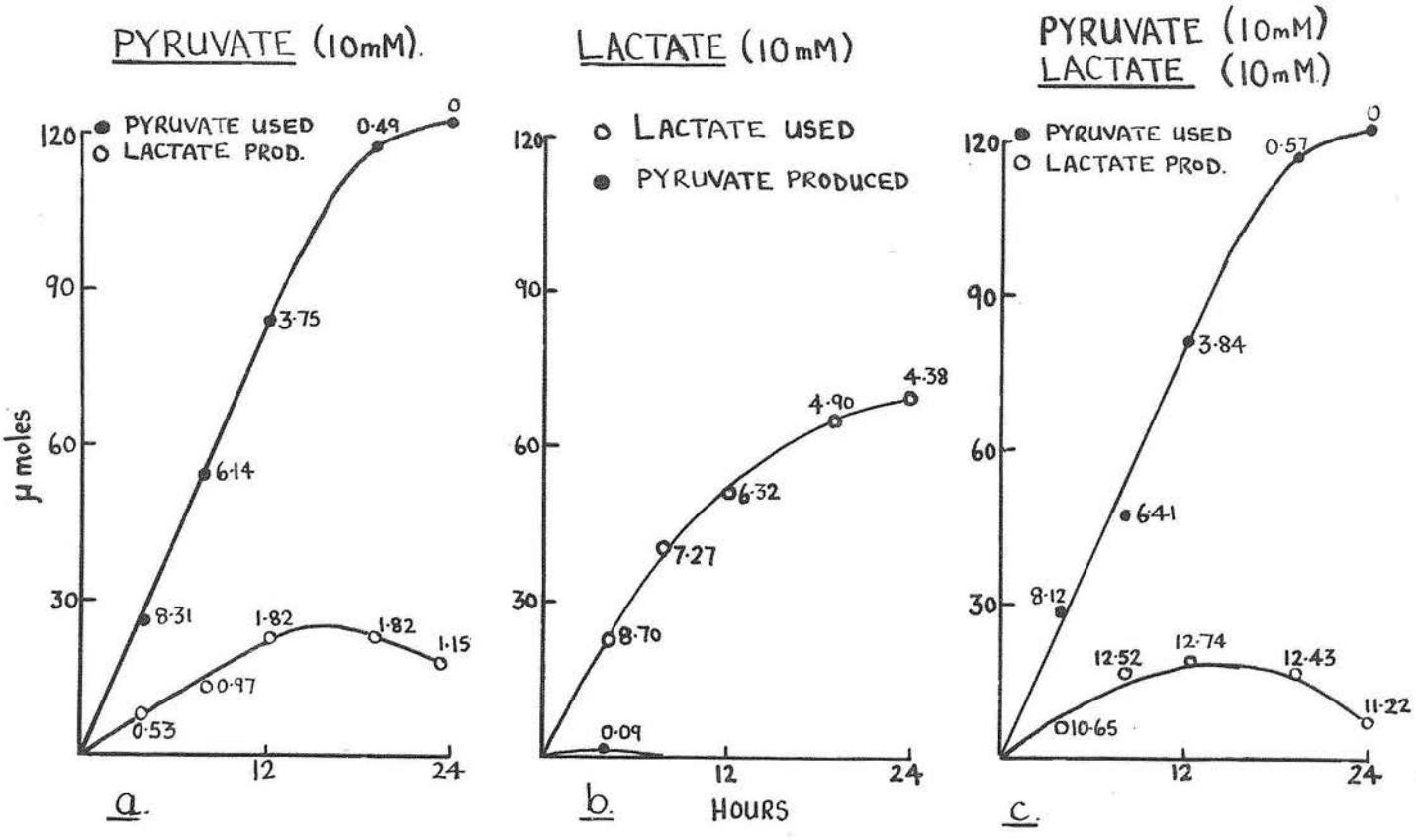
GRAPH 30

INHIBITION OF GLUCOSE UTILISATION BY PYRUVATE

TABLE 36

METABOLISM OF VARIOUS SUBSTRATES BY ROLLER BOTTLE CULTURES OVER 12 HOURS

	Substrate	Pyruvate (10 mM)	Glucose (10 mM)	Pyruvate (10mM) + Glucose (10mM)
Exp.1	Protein (mg)	10.4	10.2	10.4
	Pyruvate used (u moles)	76.3	-	62.2
	Glucose used (u moles)	-	20.6	9.1
	Lactate prod. (u moles)	19.1	1.8	26.4
	ATP (u moles) produced assuming all substrate used unaccounted for by lactate production is oxidised	858	750	
Exp.2	Protein (mg)	10.9	11.3	11.3
	Pyruvate used (u moles)	90.9	-	76.4
	Glucose used (u moles)	-	21.6	9.2
	Lactate prod. (u moles)	28.3	1.2	36.3
	ATP (u Moles)	939	800	
Exp.3	Protein (mg)	11.0	10.9	10.3
	Pyruvate used (u moles)	80.8	-	64.0
	Glucose used (u moles)	-	22.2	11.3
	Lactate prod. (u moles)	20.2	1.6	24.5
	ATP (u Moles)	909	815	



GRAPH 31a-c.

METABOLISM IN MEDIA CONTAINING PYRUVATE, LACTATE and PYRUVATE plus LACTATE. Figures denote mM. substrate in media

GROWTH EXPERIMENTS

(i) Growth in Pyruvate-containing medium

Experiments were carried out to assess growth in media containing pyruvate (10mM) but no glucose. Graph 32 shows that typically, protein increased to about the same extent as that of the control cultures in glucose-containing medium during the first day. Thereafter the growth rate declined sharply, maximum protein being attained on the third day prior to marked necrosis on the fourth day. Over several experiments the maximum protein attained varied between 1.4 and 1.7 times that initially present. On two occasions the protein content per culture reached a maximum of 1.4 times the initial on the second day, after which necrosis became apparent. The reason for this variability is obscure but is possibly related to differences in the quantity of certain precursors in the cells on inoculation. Table 37 shows an analysis of growth over four days. The most intriguing feature is that whereas protein synthesis continued, albeit at a decreasing rate for three days, ribose synthesis virtually ceased after the first day. Consequently the ratio of total ribose to protein decreased significantly from 4.6 ug. per 100 ug. to 2.9 over the experiment. Table 38 shows the results of experiments in which the acid-soluble and acid-precipitable ribose were estimated separately. From these results it is clear that the ratio of acid-soluble ribose (which is approximately 25% of the total ribose) to protein declines more rapidly than that of the acid-precipitable ribose. Within 48 hours the former ratio fell to about fifty per cent of its initial value, while the latter ratio never fell by more than thirty per cent over this period.

Although the ratio of deoxyadenosine equivalents to protein shows a decreasing trend (Tables 37 and 38) during growth in pyruvate-medium, DNA synthesis is obviously not inhibited to the same extent as that of RNA and ribose-containing nucleotides.

Table 37 shows that the quotients for pyruvate used, lactate produced and pyruvate used unaccounted for by conversion to lactate all decreased over 4 days. Pyruvate used unaccounted for by lactate production fell from 0.51 u moles per mg. protein hour over the first day to 0.21 and 0.11 during the third and fourth days respectively. Inevitably this indicates a depression of respiration rate with time. Although the quotient for lactate production decreased significantly over the four days, the proportion of pyruvate used which reappeared as lactate did not change significantly and remained at between 27 and 36%.

In summary, growth in pyruvate-containing medium in the absence of glucose is characterised by decreasing (a) rate of growth, (b) ratios of acid-soluble and acid-precipitable ribose to protein, (c) ratio of deoxyadenosine equivalents to protein, (d) quotients for pyruvate used, lactate produced, and pyruvate used unaccounted for by conversion to lactate. The only feature which growth in glucose medium over a similar time shares with the above is a decreasing quotient for lactate produced (Graph 2). It has been shown (Graph 32) that growth is not supported to any extent in the absence of a primary carbon source such as pyruvate or glucose. No decreasing ribose: protein ratios have been found in such cases.

(ii) Supplementation of pyruvate-containing medium with small quantities of ribose, adenosine and glucose

Supplementation of pyruvate-containing medium with 1mM and 5mM ribose made no difference to the growth or survival of L cells. Table 39 shows the results of an experiment where replicate cultures were sampled at 2, 3 and 4 days. Although the acid-soluble fraction of cells grown in pyruvate plus ribose showed considerable increase in ribose content over those grown in pyruvate alone, the acid-precipitable ribose was no greater, indicating that the exogenous ribose was not incorporated into ribonucleic acid.

Cell growth could not be maintained on pyruvate-containing medium supplemented with 5mM adenosine. Addition of adenosine did however permit a significantly greater protein yield and prolonged cell survival. Table 40 contains the most important information from one experiment over 8 days, replicate cultures being sampled at 2, 3, 4, 6 and 8 days. The medium was renewed on the fourth day. The results show that maximum protein yield was on the third day and was on average 2.2 times that initially present, compared with 1.7 times for growth in pyruvate alone. Necrosis was much slower when adenosine was present. By the sixth day, when cultures in pyruvate alone had undergone complete necrosis, the total protein in cultures containing pyruvate plus adenosine was on average 1.6 times that initially present. This decreased to rather less than that initially present by the eighth day. Further experiments in which the criterion was appearance of the cultures by microscopic examination confirmed that in pyruvate plus adenosine viable cells were present for 10 - 12 days whereas without adenosine cells rarely survived after five days. Table 40 shows that in the absence of pyruvate, adenosine was of no consequence as a growth substrate. The possibility was considered that the growth promoting effect apparently due to adenosine in the presence of pyruvate was in fact a result of small quantities of glucose in the adenosine preparation. Attempts to detect glucose in the adenosine preparation, using the glucose oxidase method were however unsuccessful.

A feature of metabolism in pyruvate plus adenosine is that the quotient for pyruvate unaccounted for by conversion to lactate does not undergo the very rapid and marked reduction noted for cells in pyruvate alone. Table 40 shows that in this experiment the quotient fell very slowly over the first six days and much more rapidly as necrosis became more widespread during the seventh and eighth days.

The addition of small quantities of glucose to pyruvate-containing medium greatly enhanced growth. Table 41 shows the results of a typical experiment in which replicate cultures were sacrificed at 65 hours. At that time the mean protein content per culture in pyruvate alone and pyruvate plus 0.1, 0.25 and 1.0mM glucose respectively was 1.55, 2.2, 2.5 and 2.6 times that initially present. Over this period therefore, addition of 0.25mM and 1.0mM glucose to pyruvate resulted in protein harvests which approached that in 10mM glucose alone (2.65 times initials).

The addition of similar quantities of glucose to medium without pyruvate resulted in a marked delay of necrosis, or a measure of growth. At glucose concentrations of 0.1, 0.25 and 1.0mM the cell protein present after 65 hours was respectively 0.9, 1.2 and 1.6 times the initial. This compares with 0.56 times for cultures with no glucose present. These figures illustrate the important fact that not only is growth in pyruvate-containing medium increased by small quantities of glucose, but the absolute increases are very much greater than those induced on adding similar quantities of glucose to medium without pyruvate. A further feature of growth in pyruvate-containing medium plus small quantities of glucose shown in Table 41 is the relationship between growth, the initial glucose concentration in the medium and the ribose:protein ratio noted after 65 hours. For example, cells grown in pyruvate plus 1.0mM glucose contained 4.2 s.d. 0.2 ug ribose per 100 ug. protein. This is not significantly different from that of the initials (4.3 s.d. 0.1) and cells grown in 10mM glucose (4.5 s.d. 0.2), but cells grown in pyruvate plus 0.25mM, pyruvate plus 0.10mM glucose, and pyruvate alone showed ratios of 3.8 s.d. 0.2, 3.7 s.d. 0.1 and 3.3 s.d. 0.2 respectively. It would appear therefore that inhibition of growth in pyruvate-containing medium is related to the low ribose:protein ratios which occur in the absence of glucose and the presence of pyruvate.

The results of various experiments have shown that ribose synthesis virtually ceases after 24 hours in pyruvate-containing media without glucose. It seems likely then that ribose and deoxyribose are synthesised from glucose by way of the pentose shunt pathway (page196) and that the initial increase in these compounds noted in the absence of exogenous glucose is due to the presence in the cells of small quantities of precursors such as glycogen (page102). If it is assumed that this is so and that such precursors are utilised in this manner even when small quantities of exogenous glucose are available to the cells with the pyruvate, it is possible to calculate the approximate proportion of the glucose used under these conditions which would be required to account for the ribose and deoxyribose synthesised. Calculations on this basis show that in media with pyruvate and very small concentrations of glucose, the percentage of the total glucose used required to account for ribose and deoxyribose synthesis is very much greater than when larger concentrations of glucose are present. This is shown very clearly from the results in Table 41. Analysis of the media at 65 hours showed that in all cases except the 10mM glucose cultures, no detectable glucose remained in the media. In the case of pyruvate plus 0.10mM glucose, fully 25% of the glucose used is required to account for ribose and deoxyribose synthesis during growth. The comparable figures for growth in pyruvate plus 0.25mM glucose and pyruvate plus 1.0mM glucose are 15% and 4% respectively. This compares with about 2% of the glucose used by cells in 10mM glucose without pyruvate.

The significance of these figures does not depend upon the rather doubtful assumption stated above that precursors in the cells are utilised for ribose synthesis to the same extent in pyruvate with and without glucose. If in fact, all the ribose and deoxyribose synthesis in pyruvate medium plus glucose was due to exogenous glucose, the proportions of glucose used required to account for this at concentrations of 0.10, 0.25 and 1.0mM glucose plus pyruvate would

be of the same order - approximately 29%, 17% and 5% respectively.

Table 42 contains data from an experiment to investigate in a preliminary manner the effects of exogenous pyruvate on the assimilation of 14-C glucose. It shows that cells in media with 0.10mM glucose but no pyruvate assimilated about 23% of the exogenous glucose within 24 hours. In contrast, cells in pyruvate plus 0.10mM glucose assimilated only 13% of the total exogenous glucose during this time. Glucose in the media was not measured chemically during these experiments but the large count in the medium with pyruvate after 24 hours compared with that without pyruvate indicates that the presence of pyruvate greatly reduced not only glucose assimilation but also glucose utilisation. Since rather more protein was synthesised over 24 hours when pyruvate was present, it is clear that carbon from pyruvate can readily substitute for carbon from glucose in some synthetic reactions at least.

At 55 hours necrosis was apparent in the cultures which had received 0.10mM glucose but no pyruvate. In contrast, cells in pyruvate plus 0.10mM glucose had increased and about 42% of the initial exogenous glucose was found associated with cellular material. A very substantial reduction in the total count in the medium was noted.

Sustained growth in pyruvate plus small quantities of glucose:

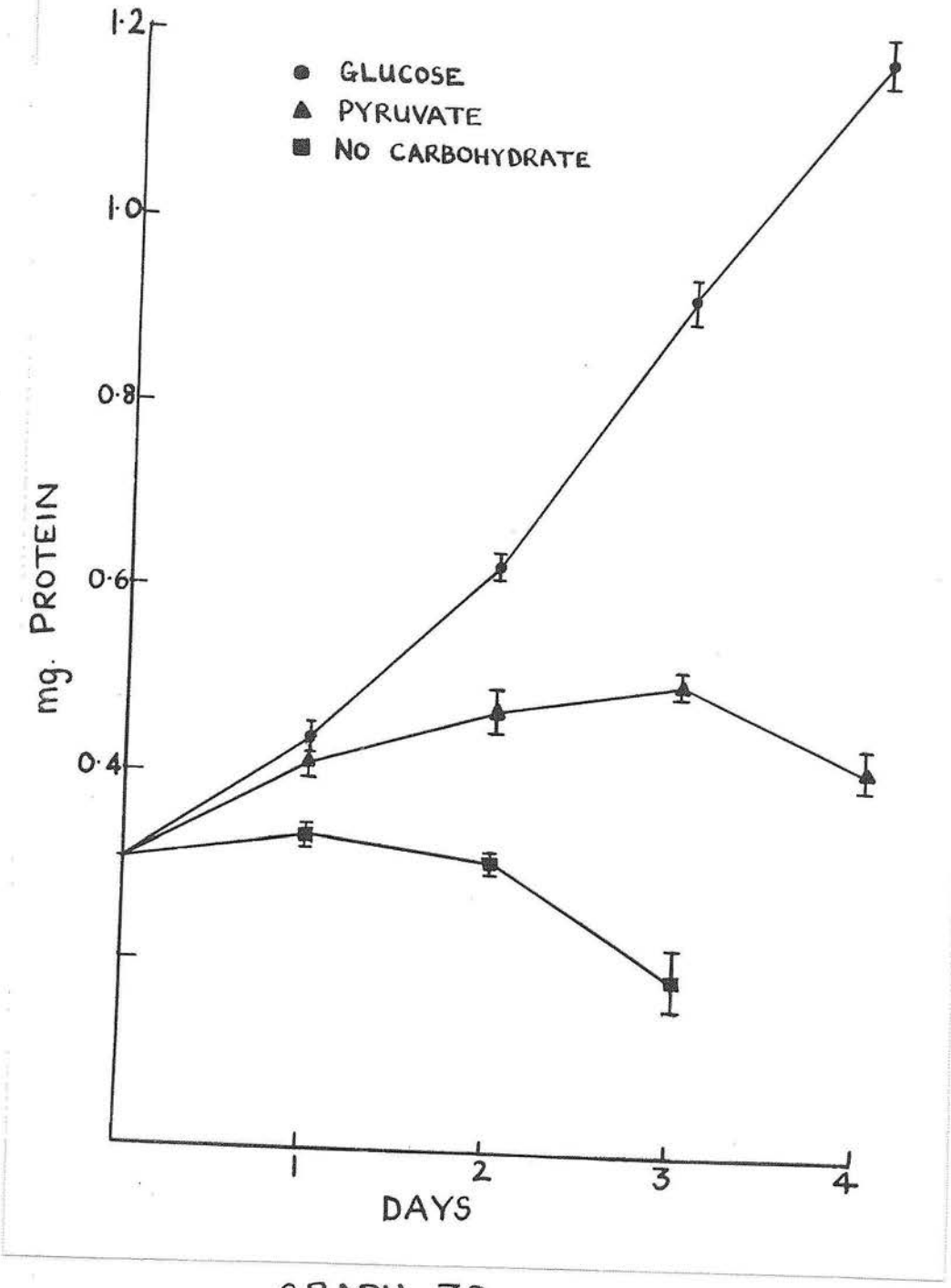
Growth was sustained for 23 days in pyruvate (15mM) plus 0.15mM glucose at rates comparable to those in 10mM glucose alone. The following schedule was adhered to:- Freshly inoculated cells received 4 ml. medium which was replaced after 48 hours and 72 hours. At 96 hours subculturing was carried out, new cultures receiving 25% of the cells. The cells increased approximately 4-fold every four days, and were subcultured four times during the 23 days. Control cultures in 0.15mM glucose alone could not maintain a

consistent growth rate on this feeding schedule which became limiting as the cultures increased in size. Control cultures were however subcultured once, and maintained themselves well.

(iii) The effects of 2:4-DNP on growth in pyruvate-containing medium

Table 43 shows that, unlike growth in glucose-containing medium, growth in medium containing pyruvate alone as the principle energy source, was not significantly influenced over 4 days by 0.05mM 2:4-DNP. Addition of 0.10 mM 2:4-DNP severely depressed but did not completely inhibit growth, cultures usually attaining a maximum protein content of 1.15 - 1.20 that initially present, by the second day, and maintaining this on the third day.

At concentrations of 0.125mM 2:4-DNP, growth was completely inhibited and by the second day necrosis was very obvious.



GRAPH 32

GROWTH. (Standard Deviations shown)

TABLE 37

GROWTH IN 10 mM PYRUVATE - CONTAINING MEDIUM

Time	0		24		48		72		96 Hours	
Number of Cultures	4		3		4		3		3	
		s.d.		s.d.		s.d.		s.d.		s.d.
Protein (u.g.)	292	10	385	21	456	18	496	12	360	17
Ribose (u.g.)	13.3	0.3	15.0	1.3	15.5	0.8	15.5	0.7	10.5	1.1
Desoxyadenosine equivalents (u.g.)	7.9	0.2	10.5	0.6	11.4	0.4	11.7	0.3	8.8	0.8
u.g. Ribose per 100 u.g. protein	4.6	0.1	3.9	0.3	3.3	0.1	3.1	0.1	2.9	0.2
u.g. Desoxyadenosine equivalents per 100 u.g. protein	2.7	0.1	2.7	0.2	2.5	0.1	2.3	0.1	2.4	0.1
u moles pyruvate used in preceding 24 hrs.			6.12		5.87		3.61		1.60	
u moles lactate produced in preceding 24 hrs.			1.95		1.62		1.22		0.44	
u moles pyruvate unaccounted for by L.A. production			4.17		4.25		2.39		1.16	
Q pyruvate unaccounted for per mg protein hour			0.51		0.42		0.21		0.11	

TABLE 38

CHANGES IN CELL RIBOSE DURING GROWTH IN PYRUVATE - CONTAINING MEDIUM WITHOUT GLUCOSE

Time	0		24		48		72 Hours	
		s.d.		s.d.		s.d.		s.d.
Experiment 1								
Protein (u.g.)	284	5	390	12	425	14	441	17
Acid soluble ribose (u.g.)	3.4	0.2	3.5	0.2	2.1	0.2	1.7	0.1
Acid-ppt. ribose (u.g.)	10.4	0.5	12.9	0.6	12.9	0.5	11.4	0.9
Acid-soluble ribose per 100 u.g. protein	1.2	0.1	0.9	0.1	0.5	-	0.4	-
Acid-ppt. ribose per 100 u.g. protein	3.7	0.1	3.3	0.1	3.1	0.2	2.6	0.2
Total ribose per 100 u.g. protein	4.9	0.1	4.2	0.1	3.6	0.2	3.0	0.2
Experiment 2		a.d.				a.d.		a.d.
Protein (u.g.)	263	8			420	4	436	15
Acid-soluble ribose (u.g.)	3.8	0.2			3.2	0	2.4	0.2
Acid-ppt. ribose (u.g.)	10.3	0.4			11.6	0.5	11.4	0.3
Acid-soluble ribose per 100 u.g. protein	1.4	0.05			0.7	-	0.6	-
Acid-ppt. ribose per 100 u.g. protein	3.9	0.1			2.8	0.1	2.6	-
Total ribose per 100 u.g. protein	5.4	0.2			3.6	0.1	3.2	-

Experiment 1 — 3 replicate cultures per day : s.d. - standard deviation from mean.

Experiment 2 — 2 replicate cultures per day : a.d. - average deviation from mean.

TABLE 39

GROWTH IN PYRUVATE - CONTAINING MEDIUM SUPPLEMENTED BY RIBOSE

Time	0		2		3		4 days	
		a.d.		a.d.		a.d.		a.d.
Protein (u.g.)								
Pyruvate (10mM)	280	8	432	14	480	17	389	7
Pyruvate (10mM) Ribose 1mM)			412	12	462	19	373	24
Pyruvate (10mM) Ribose 5mM)			430	10	449	31	372	25
Acid-soluble Ribose (u.g.)								
Pyruvate (10mM)	3.8	0.2	3.2	0.0	2.9	0.2	2.4	0.1
Pyruvate (10mM) Ribose 1mM)			8.3	0.4	8.1	0.5	8.2	0.4
Pyruvate (10mM) Ribose 5mM)			12.4	0.6	17.2	0.8	13.6	0.7
Acid-ppt. Ribose (u.g.)								
Pyruvate (10mM)	10.6	0.4	12.8	0.6	12.2	0.1	10.1	0.4
Pyruvate (10mM) Ribose 1mM)			13.2	0.4	13.3	0.2	10.0	0.3
Pyruvate (10mM) Ribose (5mM)			13.0	0.3	12.7	0.5	9.6	0.2

a.d. = actual deviation from the mean of two cultures.

TABLE 40

GROWTH IN PYRUVATE - CONTAINING MEDIUM SUPPLEMENTED BY ADENOSINE

Time	0		2		3		4		6		8 days	
Protein (u.g.)		a.d.		a.d.		a.d.		a.d.		a.d.		a.d.
Pyruvate (10mM) Adenosine (5mM)	310	9	596	22	682	12	640	21	496	4	278	22
Pyruvate (10mM)			489	15	523	12	396	19	63	32	-	
Adenosine (5mM)			242	14	171	25	-					
Neither			230	6	149	17	-					
Pyruvate used unaccounted for by conversion to lactate (μ moles/mg. protein hr.)			0-2		2-3		3-4		4-6		6-8 days	
Pyruvate 10mM) Adenosine (5mM)			0.51		0.58		0.48		0.39		0.19	
Pyruvate			0.40		0.23		0.14		0.08			

TABLE 41

GROWTH OVER 65 HOURS IN MEDIA CONTAINING PYRUVATE AND GLUCOSE

Substrate (mM) Pyruvate	Glucose	Protein (u.g.)	Ribose (u.g.)	Deoxyadenosine (u.g.)	Prot.(65hr) / Prot.(init)	u.g.Ribose per 100 ug Prot.	u.g.deoxyad per 100 ug Prot.	u moles Glucose required for Pentose Synthesis	% Glucose used for Pentose
		342 (Init) s.d.11	14.6 0.5	9.2 0.4		4.25 0.1	2.7 0.1	-	-
-	-	192 s.d. 9	8.7 0.8	4.9 0.2	0.56	4.5 0.2	2.5 0.0		
-	0.1	315 s.d. 9	13.4 0.8	9.2 0.3	0.92	4.3 0.1	2.9 0.0		
-	0.25	410 s.d.15	16.8 0.6	11.1 0.5	1.20	4.0 0.2	2.7 0.1	0.02	2%
-	1.0	552 s.d.16	23.0 2.0	15.2 .04	1.60	4.4 0.2	2.75 0.0	0.07	2%
-	10.0	910 s.d.29	40.8 2.1	24.7 1.10	2.65	4.5 0.5	2.7 0.1	0.22	2%
10.0	-	516 s.d.14	17.1 1.1	11.8 0.4	1.55	3.3 0.2	2.3 0.0	-	-
10.0	0.1	751 s.d.17	28.2 1.2	20.8 0.8	2.20	3.7 0.1	2.7 0.2	0.10	25%
10.0	0.25	860 s.d.26	34.2 0.4	24.7 0.8	2.52	3.8 0.2	2.8 0.1	0.16	16%
10.0	1.00	884 s.d.16	37.5 3.0	24.0 0.6	2.58	4.2 0.2	2.7 0.0	0.18	4%

TABLE 42

THE EFFECT OF PYRUVATE ON THE ASSIMILATION OF 14-C GLUCOSE

	24 Hours					55 Hours				
	u.g. Protein per flask	Counts per 1000 secs.	u.moles glucose assim.	% assim.	Total counts in media per 1000 seconds	u.g. Protein per flask	Counts per 1000 secs.	u.moles glucose assim.	% assim.	Total counts in media per 1000 seconds
Glucose (0.1mM)	370 ±12	1703 ±142	0.094 ±.007	23	403 ±42	282 ± 4	1337 ±32	0.074 ±0.002	18	563 ±32
Glucose (0.1mM) + Pyruvate (10mM)	432 ±10	958 ±25	0.053 ±0.002	13	4022 ±203	604 ±21	3044 ±150	0.168 ±0.007	42	858 ±41

Initial protein = 282 u.g. ± 11 per flask

1 u mole glucose = 18209 counts per 1000 seconds

2 replicate cultures measured at each point

TABLE 43

EFFECT OF 2:4 DNP ON GROWTH IN PYRUVATE - CONTAINING MEDIUM

2:4-DNP	-		0.05		0.10		0.125 mM	
Protein (u.g.)		s.d.		s.d.		s.d.		s.d.
Initial	302	9						
48 Hours	462	19	449	9	348	7	201	11
72 hours	497	10	482	4	333	12	-	
96 Hours	365	10	357	16	214	8	-	

DISCUSSION

Pyruvate can enter and undergo metabolism in a wide range of mammalian tissues. These include rat diaphragm muscle and adipose tissue (Beloff-Chain et al, 1962) sheep thyroid slices (Dumont, 1956), rat heart (Newsholme and Randle, 1962), bull sperm (Melrose and Terner, 1953) and ascites tumour cells (Emmelot and Bos, 1959). The foregoing experiments with strain L fibroblasts show that under manometric and short term growth conditions in stationary and rolling cultures pyruvate is readily removed from the medium and undergoes assimilation, oxidation to carbon dioxide and reduction to lactic acid which appears in the medium. Pyruvate appears to have little difficulty in reaching the metabolic systems within the cell, and the maximum respiration rate under manometric conditions is achieved with external concentrations as low as 0.5mM.

The respiration rate (24.2 s.d. 1.0) is significantly greater than that for glucose (18.8 s.d. 1.3), a finding which is compatible with the conclusion (page[31]) that the rate for glucose respiration is conditioned to some extent by "competition" with glycolytic processes for ADP. The increased rate for pyruvate respiration may therefore indicate that when the glycolytic system is not essential for the entry of oxidisable substrates into the TCA cycle, the adenine nucleotides concerned in glycolytic substrate level phosphorylations become available for respiratory chain phosphorylations. The appearance in the medium of lactate corresponding to 25 - 36% of the total pyruvate used has been noted under manometric and growth conditions in both stationary and rolling cultures and appears to be an intrinsic feature of the pyruvate metabolism of these cells. The production of lactate from pyruvate has been observed in several animal tissues and bacteria under anaerobic conditions (Krebs, 1937; Krebs and Johnson, 1937), and in bull sperm under aerobic conditions (Melrose and Terner, 1953) when as much as 70% of the pyruvate used was reduced to lactate. The most likely explanation for this

phenomenon is that the oxidation of pyruvate is accompanied by an $NADH/NAD^+$ ratio which is significantly poised for the reduction of a proportion of the pyruvate entering the cell. In the absence of pyruvate, lactate is metabolised by L cells, the respiration rate (20.4 s.d. 0.3) being greater than that for glucose, but less than the rate for pyruvate. The difference in the rates of pyruvate and lactate seems unlikely to be related to selective membrane permeability for these substrates. There is no available evidence to indicate that specific transport mechanisms exist for lactate and pyruvate and Giebbels and Passou (1960) have concluded that monocarboxylic acids enter erythrocytes by simple diffusion across the lipoid phase as undissociated molecules. Apart from the fact that there are no important physico-chemical differences between the two molecules which might be expected to influence their rate of entry by diffusion, their pK values indicate that if anything, lactate might have an advantage in that at pH 7.4 the proportion of undissociated molecules is approximately ten times that of pyruvate. Furthermore, that the membrane is readily permeable to lactate is suggested by the greatly increased respiration rate on uncoupling respiratory chain phosphorylations. Since lactate respiration is unlikely to be limited by its ability to enter the cell and since there is presumably as much ADP available for respiratory chain phosphorylations as when pyruvate is the exogenous substrate, the limiting factor may well be the rate at which lactate can be oxidised to pyruvate, a process which is clearly dependent upon a suitable $NAD^+/NADH$ ratio. A consequence of the suggestion that with pyruvate as exogenous substrate respiration rate is limited by phosphorylations, whereas with lactate it is limited by the rate of pyruvate production from lactate, is that in the former case the components of the respiratory chain might be expected to be more reduced than in the latter (Chance and Williams, 1955; Hess, 1961). The nature of possible feedbacks with the NAD linked lactic dehydrogenase must

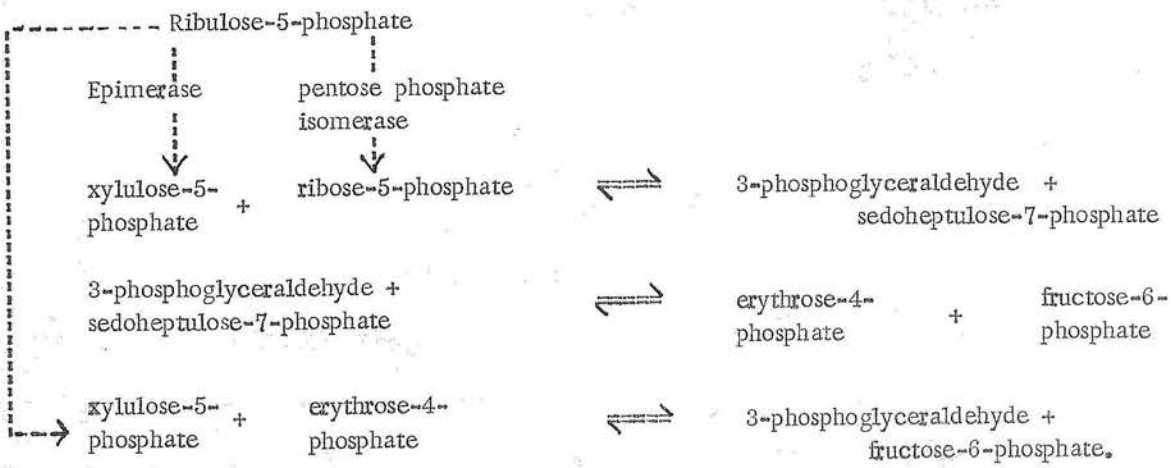
however be regarded as obscure, as is the relationship between mitochondrial and extramitochondrial NAD systems, and the entire concept of NAD^+/NADH ratios as regulatory mechanisms for metabolism (Klingenberg, 1964). It is of note that uncoupling of oxidative phosphorylations increases the respiration rates of both pyruvate and lactate, a process which supposedly leads to a more oxidised state of the respiratory carriers. This is compatible with the observation that uncoupling of pyruvate oxidation significantly decreases the rate of lactate production. Alternative explanations are however possible, and this may merely reflect competition for substrate between the non-phosphorylating oxidative pathway and the reductive reaction. The phenomenon seems worthy of spectrophotometric investigation. When pyruvate and lactate are added together to cells, there is net decrease in pyruvate and net increase in exogenous lactate until the pyruvate is completely used, after which the lactate is slowly utilised. This supports the general conclusion that the related objectives of optimum respiration rate and optimum NAD^+/NADH ratio for cell maintenance and growth are achieved more readily by the oxidation of pyruvate to carbon dioxide, linked with the reduction of pyruvate to lactate, than by the oxidation of lactate to pyruvate and its subsequent oxidation to carbon dioxide. At present no more precise analysis of the findings is possible.

Manometric and roller bottle experiments have shown that exogenous pyruvate at concentrations of from 10mM to about 0.5mM effectively reduces the utilisation of exogenous glucose by more than fifty per cent. This is in agreement with the findings of Newsholme and Randle (1962) who noted that in addition to pyruvate, the respiration of fatty acids inhibited glucose utilisation in rat diaphragm. These authors noted that the inhibition operated at the phosphorylation of fructose-6-phosphate to fructose-1:6-diphosphate. The manner in which inhibition of phosphofructokinase can backtrack to inhibit

the hexokinase reaction has already been discussed (page129). It will be recalled that the purified enzyme is inhibited by high concentrations of ATP, but is relatively insensitive to all other members of the EMP pathway including pyruvate and lactate, but excluding its product fructose-1:6-diphosphate which produces a stimulatory effect. The latter findings suggest that inhibition of the enzyme during pyruvate respiration is not primarily the result of a build-up of EMP pathway intermediates due to competition between exogenous pyruvate and pyruvate produced from glucose for entry into the respiratory cycle, although this may occur to some extent. A high ATP:ADP ratio in the cytoplasm appears to be a more likely reason: this is in accord with the idea already expressed that when pyruvate is available for oxidative decarboxylation without the essential functioning of the EMP pathway, respiratory chain phosphorylations have a priority claim on ADP (Gatt, 1957). A point of some importance is that inhibition of phosphofructokinase need not induce a similar inhibition of glucose metabolism through the hexose monophosphate shunt pathway (HMP) (Horecker and Mehler, 1955). This is a cyclic mechanism of glucose-6-phosphate metabolism through 6-phosphogluconate and ribulose-5-phosphate, the sum of these reactions being

$$\text{glucose-6-phosphate} + 2\ NADP^{+} + H_2O \rightleftharpoons \text{ribulose-5-phosphate} + 2\ NADPH + CO_2$$

The reaction sequence from ribulose-5-phosphate may then proceed



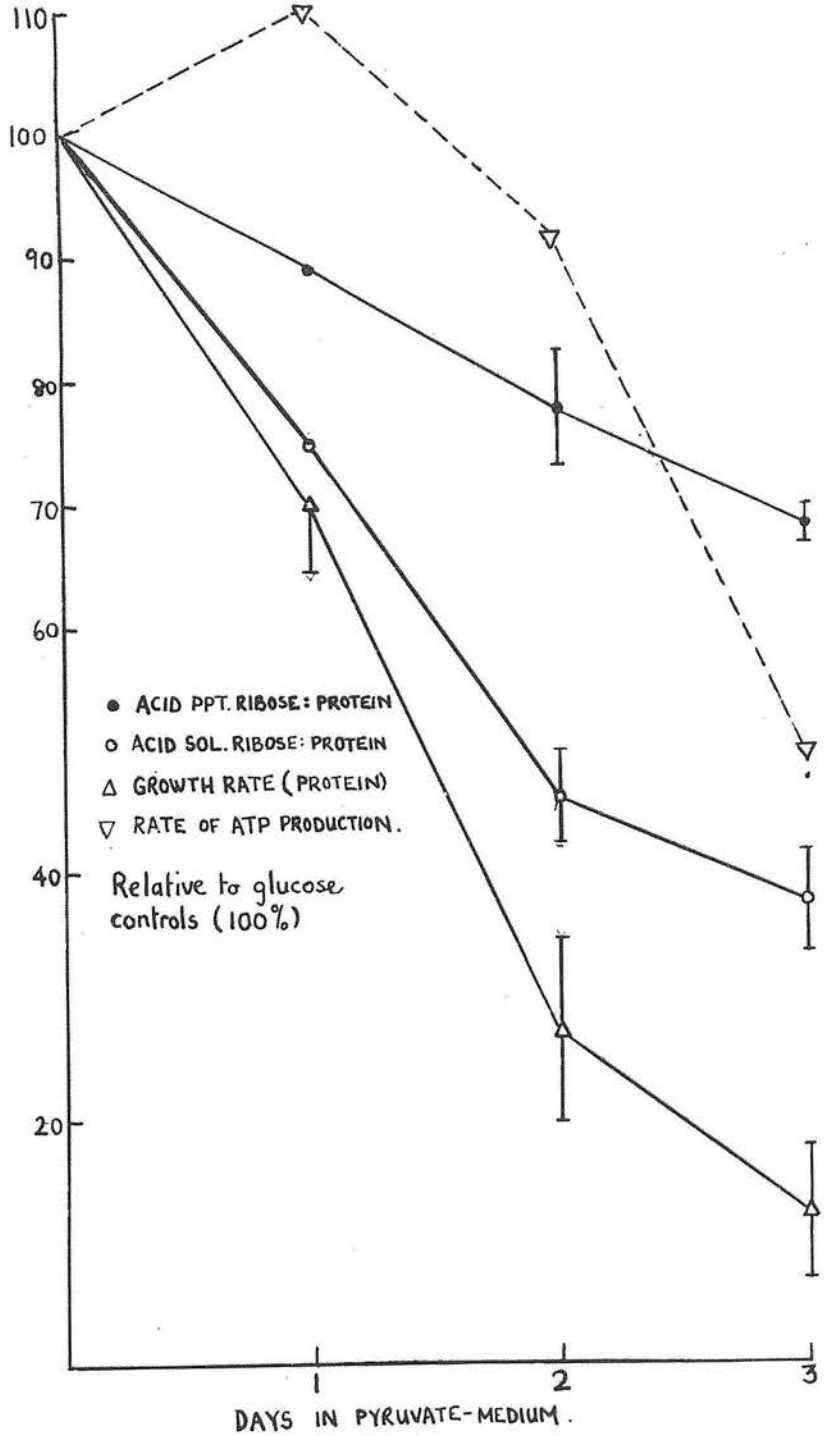
Glucose may therefore circumvent the phosphofructokinase reaction and participate in assimilatory, oxidative and glycolytic reactions. Although the HMP has not been demonstrated in L cells, it appears to be of ubiquitous occurrence and has been found in micro-organisms, a very wide variety of mammalian tissues and HLM tissue culture cells (Crockett and Leslie, 1963). The contribution of the HMP to carbohydrate metabolism has been evaluated almost solely through the use of specifically labelled glucose and depends upon assumptions which are not entirely satisfactory (Woods, 1955; Utter, 1958). Assessments of the percentage of glucose passing through the shunt must be regarded as semi-quantitative and differ widely from tissue to tissue. In mammary gland it has been calculated to be as high as 60% (Abrahams et al, 1954). Considerable evidence indicates that the activity of the pathway varies with the $NADP^+$/NADPH ratio and Ashan et al (1960) have suggested that one of the main functions of the pathway is to provide NADPH which is essential for fatty acid synthesis. Without 14-C glucose experiments it is not possible to evaluate the proportions of glucose utilised by L cells in the presence of exogenous pyruvate which are dissipated by oxidative, assimilative and glycolytic routes. The following points are of some relevance:- When glucose is present, the rate of pyruvate utilisation is slightly but consistently reduced in roller bottle experiments, and in manometric experiments the respiration rate when both substrates are added is in most cases, but not always, less than the rate with pyruvate alone. Related to this is the fact that the presence of glucose always leads to an increase in lactate production. The slight reduction in respiration rate when glucose is present may be explained in terms of a limited involvement of ADP in the EMP pathway despite the availability of exogenous pyruvate for respiration. This would suggest that the increased lactate production is due in part at least to glucose being metabolised through the EMP, rather than increased reduction of exogenous pyruvate. Before leaving the subject of suppression of glucose utilisation by exogenous pyruvate, it may be pointed out

that although this occurs with concentrations of pyruvate as low as 0.5mM, this is a considerably greater concentration than that accumulating in the medium during monolayer growth experiments in glucose-containing medium (Graph 1).

* * * * *

Despite its efficiency as a substrate for respiration, pyruvate is inadequate as a growth substrate, and L cells in pyruvate-containing medium show the following features over four days:- (1) The growth rate (protein) decreases after the first day and stops on the third, after which necrosis becomes apparent. (2) The quotient for pyruvate utilised unaccounted for by lactate production decreases from the first day and on the fourth day is a mere 20% of its value on the first. (3) The rate of lactate production per mg. protein hour decreases in a manner which corresponds to that for (2) above. (4) The ratios of both acid-soluble and acid-precipitable ribose to protein decrease from the first day, the decrease being more obvious in the first case. (5) The ratio of deoxyadenosine equivalents to protein shows a decreasing trend.

These departures from steady state metabolism are expressed graphically overleaf. The primary factor in unbalancing growth and metabolism in this manner appears to be the inability of the cells to synthesise ribose-5-phosphate from pyruvate; in this event the subsequent inhibition of nucleic acid and nucleotide synthesis would rapidly lead to inhibition of respiration and growth. Certain thermodynamic difficulties are involved in the synthesis of pentose phosphates from pyruvate by reversal of glycolysis and the HMP. These apply particularly to the phosphofructokinase and pyruvate kinase reactions. The phosphofructokinase reaction is virtually irreversible and the formation of fructose-6-phosphate from fructose-1:6-diphosphate entails hydrolysis by a



Information from Tables 37 and 38.

specific phosphatase which liberates Pi but does not regenerate the pyrophosphate bond to form ATP. In the case of the pyruvate kinase the equilibrium constant is 2×10^{-3} in the direction of pyruvate and ATP (Meyerhoff, 1949) and therefore highly unfavourable for the formation of phosphoenolpyruvate. In liver and to a much lesser extent in muscle, reversal of glycolysis may occur by the "dicarboxylate shunt" which circumvents the pyruvate kinase reaction by converting pyruvate and carbon dioxide to oxaloacetate which is decarboxylated by oxaloacetic decarboxylase to phosphoenolpyruvate (Krebs, 1954). This pathway which is best authenticated for liver, appears to be related to the physiological role and the glycogen metabolism of the tissue and is by no means ubiquitous. The inability of strain L cells to synthesise ribose from pyruvate may indicate that the pathway, if present, is of little importance. Furthermore, an unfavourable factor for any likely enzymic adaptation to the replacement of glucose in the medium by pyruvate is the rapidity with which the respiration rate and therefore rate of ATP production decreases. This decrease is the anticipated consequence of the fall in adenine nucleotides and cofactors, implied by the marked decrease in acid-soluble ribose:protein ratio.

If the assumption is made that approximately 20% of the total pyruvate unaccounted for by lactate production is assimilated, the average rates of ATP production over 4 days are 6.1, 5.1, 2.7 and 1.4 u moles respectively per mg. protein hour. This assumption on pyruvate assimilated is based on (a) the fact that manometric experiments with pyruvate as substrate, and growth experiments with 14-C glucose and pyruvate (Table 42) show that pyruvate is in fact assimilated, and (b) it seems reasonable to conclude that the extent to which pyruvate is assimilated can be no greater than that determined for 14-C glucose assimilation (page 85) under optimum growth conditions. The figure of 20% of pyruvate used unaccounted for by lactate production should be regarded as the maximum for pyruvate assimilated. Certainly no more than this can be

assimilated on the first day in pyruvate-containing medium, and considerably less may be assimilated on subsequent days. Consequently the calculated rates shown above of ATP production from pyruvate during four days should be taken as the minimum rates. Over the first day the rate is approximately the same as that calculated for growth under similar conditions with glucose as substrate (page 162), and it is of interest that on the second day it is at least 80% of the glucose rate, although the growth rate in pyruvate is now very much lower. This latter fact suggests that it is the reduced synthesis of ribose-5-phosphate rather than the fall in respiration which initiates inhibition of growth at this stage. The extent of mandatory coupling between ribonucleic acid metabolism and protein synthesis is a matter of dispute (Reid, 1960). L cells metabolising pyruvate show a very limited synthesis of protein in the absence of net increase of ribonucleic acid; this inhibition of protein synthesis is probably related not only to impaired ribonucleic acid synthesis but to decreases in ribose-containing cofactors important in synthetic reactions.

Ribose added to pyruvate-containing medium readily enters the cells, as shown by a large increase in acid-soluble ribose, but does not influcence growth rate, acid-precipitable ribose, or the quotients for pyruvate "unaccounted for by reduction". As ribose requires to be phosphorylated to ribose-5-phosphate before it can enter the sequence leading to the biosynthesis of nucleotides (Kornberg et al, 1955) it appears that this reaction cannot occur in L cells. Although Eagle, Barban, Levy and Schulze (1958) claim that ribose permits limited growth of certain human cell lines, the findings of most other authors (Harris and Kutsky, 1953; Chang and Geyer, 1957; Bailey, Gey and Gey, 1959) show that its ability to support survival or growth is negligible.

Adenosine differs from ribose in that it markedly increases the quantity and survival time of cells in pyruvate-containing medium. A finding of some interest is that adenosine offsets the marked decrease in pyruvate used unaccounted

for by reduction with time in pyruvate-containing medium. This latter observation suggests that adenosine is readily phosphorylated and can enter the adenine nucleotide pool; and gives some support to the idea already expressed that the initial decrease in respiration rate in pyruvate-containing medium is primarily a consequence of a fall in adenine nucleotides - AMP, ATP and ADP. The failure of adenosine plus pyruvate to support long term growth is obscure and should be investigated further. If adenosine can be phosphorylated to adenosine-5-phosphate as suggested above, simple cleavage of the glycosidic bond by pyrophosphate to form the base and 5-phosphoribosyl-1-pyrophosphate would open the way to synthesis of the various other nucleotides by reaction of the relevant bases with the 5-phosphoribosyl-1-phosphate. Whether the failure to support growth is due to limiting kinetics of these reactions or some other reason remains in doubt.

Glucose added to pyruvate-containing medium at concentrations sufficiently small to make little contribution to energy metabolism considerably stimulates both growth rate and the final cell harvest. Growth under such conditions is characterised by significantly greater ribose:protein ratios than those observed after some time in pyruvate-containing medium without glucose; this supports the general conclusion that whereas pyruvate may be oxidised efficiently providing sufficient ATP for the metabolic and synthetic reactions of the cell, glucose is essential for the synthesis of pentose-5-phosphates before balanced growth can be achieved. Assuming that the ribose synthesised by cells in glucose plus pyruvate medium is almost entirely derived from glucose it has been shown that there is an inverse relationship between the exogenous glucose concentration and the proportion converted to ribose. For example, when the initial glucose concentration was 0.1mM, about 25% could be accounted for by ribose synthesis. At 1.0mM glucose, the proportion was only 4%. These figures (Table 41) give no indication of the percentage of the total glucose used

metabolised through the shunt, and the simplest hypothesis to account for them is competition for the ribose-5-phosphate formed from ribulose-5-phosphate, between synthetic reactions and the reaction with xylulose-5-phosphate to form 3 phosphoglyceraldehyde and sedoheptulose-7-phosphate; the former reactions having the priority claim.

Preliminary experiments with 14-C glucose at concentrations well below the km for the respiratory and glycolytic systems have shown that exogenous pyruvate inhibits the assimilation of 14-C into cellular material by at least 50% over the first twenty-four hours. As growth during this period is slightly greater in the presence of pyruvate than in its absence, it is clear that pyruvate carbon must readily substitute for glucose carbon in some synthetic reactions, possibly the synthesis of fats through acetyl-Co-A and of protein by the formation of non-essential amino acids. It is apparent however that the sparing effect of pyruvate on glucose utilisation results in a significantly greater proportion of glucose being eventually assimilated. In the absence of pyruvate the glucose (0.1mM) appears to be virtually removed from the medium within 24 hours, the 14-C (23%) found associated with the cells being the maximum assimilated. When pyruvate is present, the glucose is utilised slower and by 55 hours when little or no exogenous glucose remains, as much as 42% of the total glucose is associated with cellular material. The distribution of this within the cell remains to be determined. These experiments support the hypothesis that the observed ability of cells to grow for long periods of time in pyruvate plus small quantities of glucose at rates approaching those in glucose medium is due to reduction in the rate at which glucose can enter respiratory and other reactions through which pyruvate can be metabolised - with the result that the glucose is metabolised through essential pathways such as the hexose monophosphate shunt at slower rates.

Dinitrophenol:

The inability of 0.1mM dinitrophenol to suppress completely the small measure of growth which occurs in pyruvate-containing medium appears to support the conclusion from the glucose experiments (page 173) that this concentration does not entirely uncouple respiratory chain phosphorylations in cells metabolising at pH 7.4 in bicarbonate-buffered medium.

SECTION 3

EXPERIMENTS WITH SUCCINATE AS SUBSTRATE

EXPERIMENTAL

MANOMETRIC EXPERIMENTS

The respiration rate of L cells was not appreciably stimulated by succinate at concentrations normally used for glucose, pyruvate and lactate metabolism, that is 5mM to 10mM. Graph 33, however, shows that a linear relationship exists between respiration rate and external succinate concentration to 42.5mM at least. Above this concentration, respiration did not proceed at a linear rate with time and cell deaths increased, possibly as a result of ionic imbalance. Even at 42.5mM succinate, the increase in respiration (25%) was small in comparison to that induced by pyruvate, lactate and glucose (page 180).

Graph 34 shows that where the plasma membrane was absent or mildly damaged, as in the case of mitochondria and cells damaged by freeze-thawing, (see pages 52-53) the QO_2 reached its maximum value at external succinate concentrations much lower than those required for intact cells. In the case of mitochondria this occurred at about 2.5mM succinate, and maximum respiration of damaged cells was induced by 5mM succinate. These findings suggested that the respiration of succinate by intact cells was limited by its ability to permeate the plasma membrane.

Table 44 contains results from experiments to test the hypothesis that a lowering of external pH with a concomitant increase in the proportions of the undissociated and partly ionised forms of 30mM succinate would be reflected in increased respiration rates due to the more ready entry of the substrate in those forms. In all the experiments undertaken, the QO_2 at pH 6.9 was 6 - 10% higher than at pH 7.6 and 7.2. At pH values below 6.6 the respiration rate was lower than at 7.2 - 7.6. This was also evident in endogenous controls and may indicate an inhibitory effect of low pH on specific enzyme systems. Previous experiments (Graph 19) had indicated that the slight inhibition of glucose respiration noted at pH 6.6 did not prevent considerable stimulation of respiration on the addition of 2:4-DNP at this pH. Uncoupling was therefore considered as

a possible means of assessing the availability of succinate at the oxidation sites within the cell at different pH values.

Graph 35 shows that 0.033mM 2:4-DNP increased the respiration rate of cells metabolising 13, 30 and 42.5mM succinate at pH 7.4 by essentially the same factor as it increased the rate of endogenous respiration - approximately 2 ultrs. O_2 per mg. protein hour. At pH 7.4 therefore there is no indication that this concentration of uncoupler increases the rate of succinate oxidation at all. In contrast to its effects at pH 7.4, addition of succinate to cells at pH 6.3 was not accompanied by increases in respiration proportional to external succinate concentrations. For example, at 13mM succinate, respiration was stimulated by about 15% but concentrations of succinate up to 42.5mM failed to increase this further. This again appears to indicate a limiting effect of low pH on respiration rate. On addition of 0.033mM uncoupler, respiration at pH 6.3 was greatly stimulated to rates which were directly related to external succinate concentration and considerably greater than those attained at pH 7.4. For example, the 2:4-DNP-induced rate of respiration in 42.5mM succinate at pH 6.3 was almost twice that at pH 7.4.

The most obvious hypotheses to explain the differences between 2:4-DNP stimulated respiration rates at pH 6.3 and 7.4 shown in Graph 35 are as follows:-

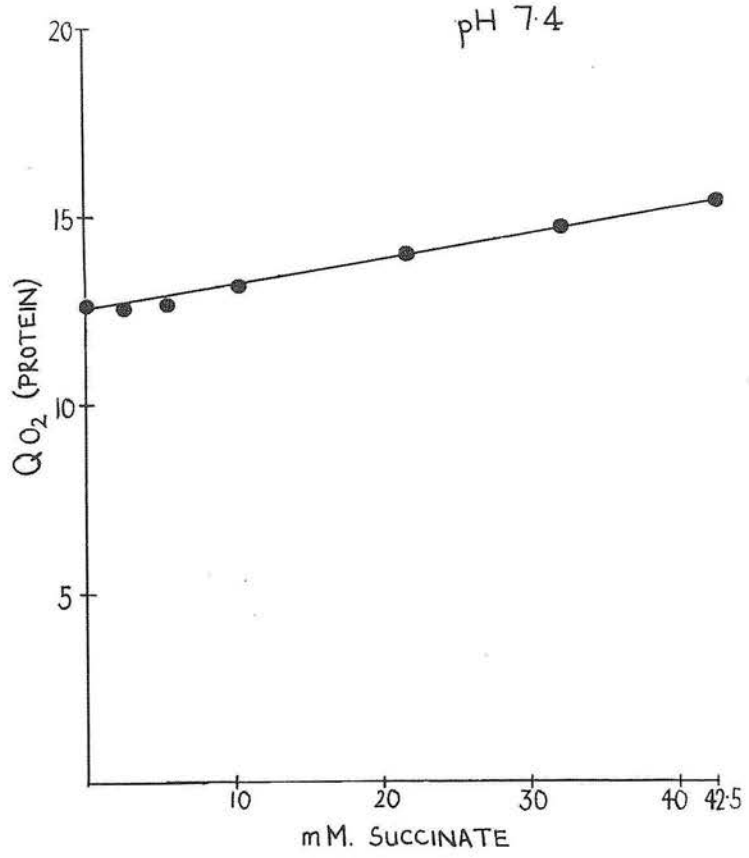
(i) Increased 2:4-DNP entry/activity at the low pH
(ii) Increased rate of entry of succinate at the low pH
(iii) (i) and (ii).

The results of experiments to resolve this are shown in Graph 36. The effects of 2:4-DNP at concentrations between 0.01mM and 0.40mM were examined on cells in 40mM. succinate at pH 6.3 and pH 7.4. The results show that at pH 7.4 the greatest stimulation of respiration was induced by 0.1mM 2:4-DNP, succinate resembling pyruvate and lactate in this respect. The QO_2 induced by 0.1mM uncoupler was however no more than 25% greater than the respiration rate in the absence of the uncoupler. Endogenous respiration was stimulated maximally

by 0.1mM also, and it is clear that this alone could account for the observed 2:4-DNP induced stimulation of respiration in 40mM succinate.

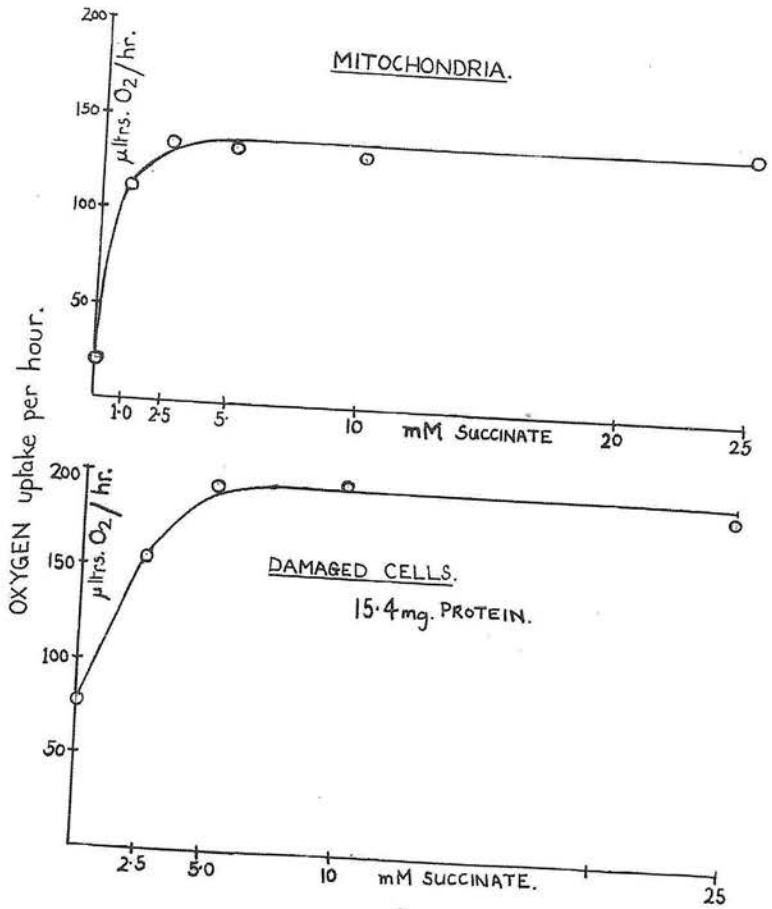
The maximum stimulation of respiration of succinate at pH 6.3 was induced by about 0.03mM 2:4-DNP. With an external succinate concentration of 40mM, this concentration of uncoupler increased respiration rate 2.5-fold. At greater 2:4-DNP concentrations this rate was severely depressed, following the patterns observed for glucose, lactate and pyruvate respiration with increasing 2:4-DNP concentrations.

Pyruvate was used as a substrate in experiments to assess the effects of pH on 2:4-DNP entry/activity where substrate was unlikely to be the limiting factor for respiration. (Graph 27 shows that the QO_2 for cells oxidising pyruvate at pH 7.4 reaches a maximum value with external concentrations of only 0.5mM pyruvate.) Graph 37 shows that 0.1mM 2:4-DNP stimulated pyruvate respiration maximally at pH 7.4, and about 0.03mM did the same at pH 6.3. At both pH values the maximum 2:4-DNP stimulated respiration rate was approximately the same.



GRAPH 33

RESPIRATION RATES WITH SUCCINATE



GRAPH 34

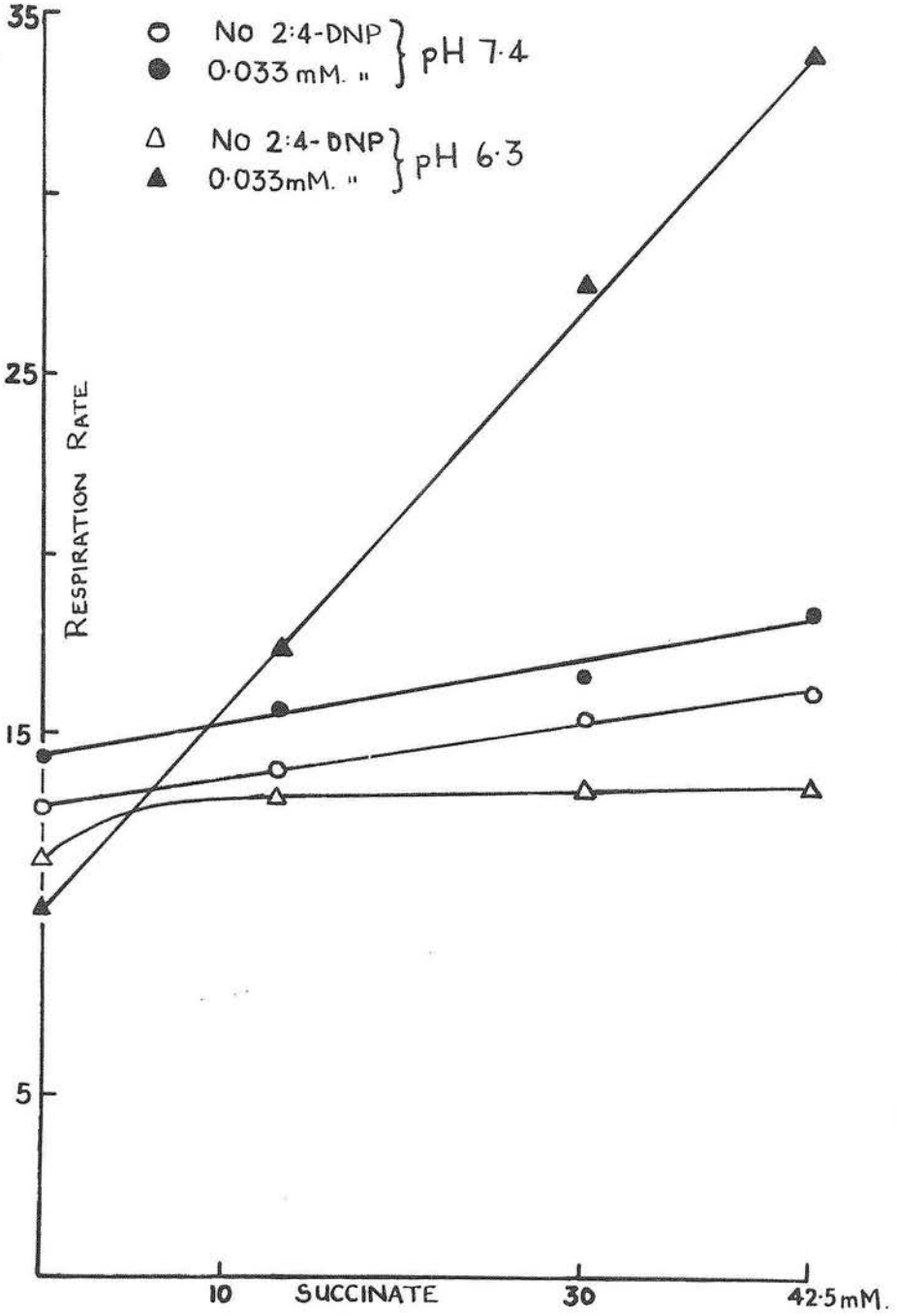
RESPIRATION OF MITOCHONDRIA AND CELLS DAMAGED BY FREEZE-THAWING.

TABLE 44

EFFECT OF pH ON SUCCINATE RESPIRATION

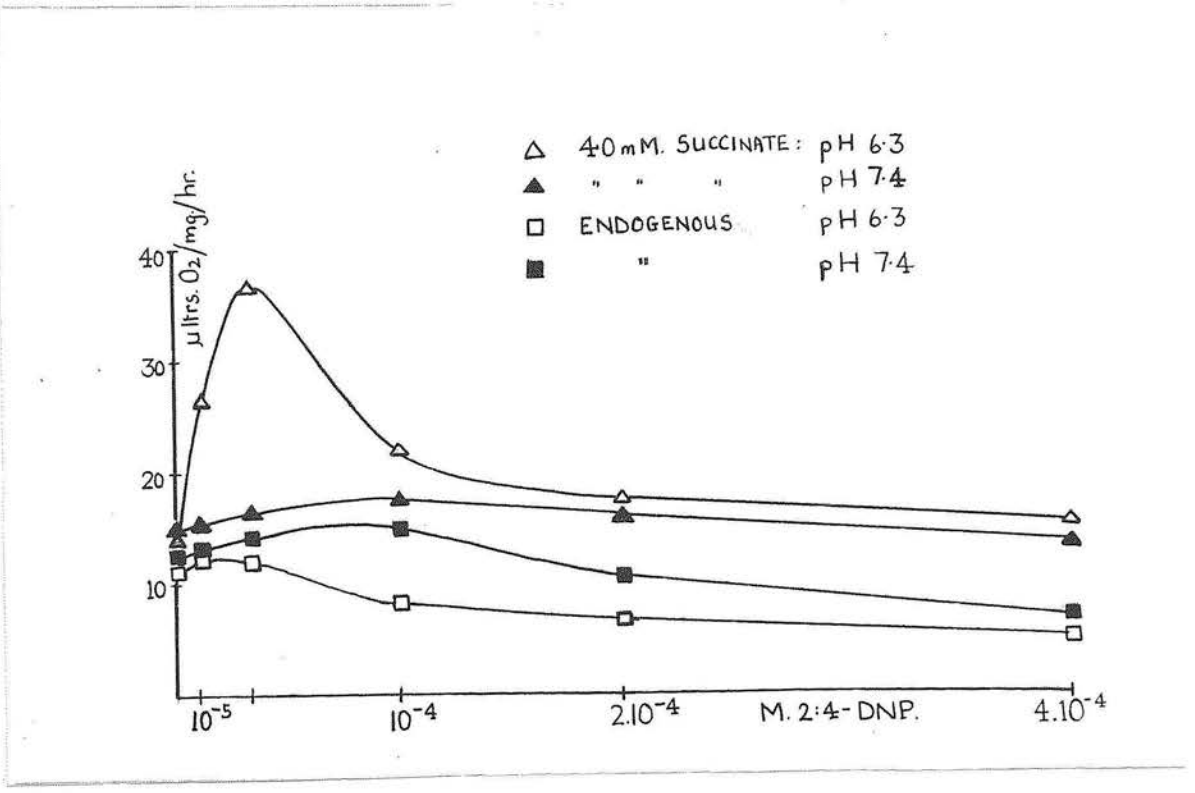
Respiration Rate

pH	7.6	7.2	6.9	6.6	6.3
30 mM Succinate					
Exp. 1	14.6	14.2	15.8	15.0	13.2
Exp. 2	13.9	15.6	15.8	14.9	-
	14.4	14.4	16.2	11.1	-
Exp. 3	16.0	13.8	16.9	-	14.9
Exp. 4	13.6	14.2	14.9	-	12.3
Mean:	14.5	14.4	15.9	13.7	13.5
Endogenous					
Exp. 1	13.1	13.1	12.8	12.5	11.7
Exp. 2	12.6	11.7	13.0	12.6	11.3
Mean:	12.8	12.4	12.9	12.5	11.5

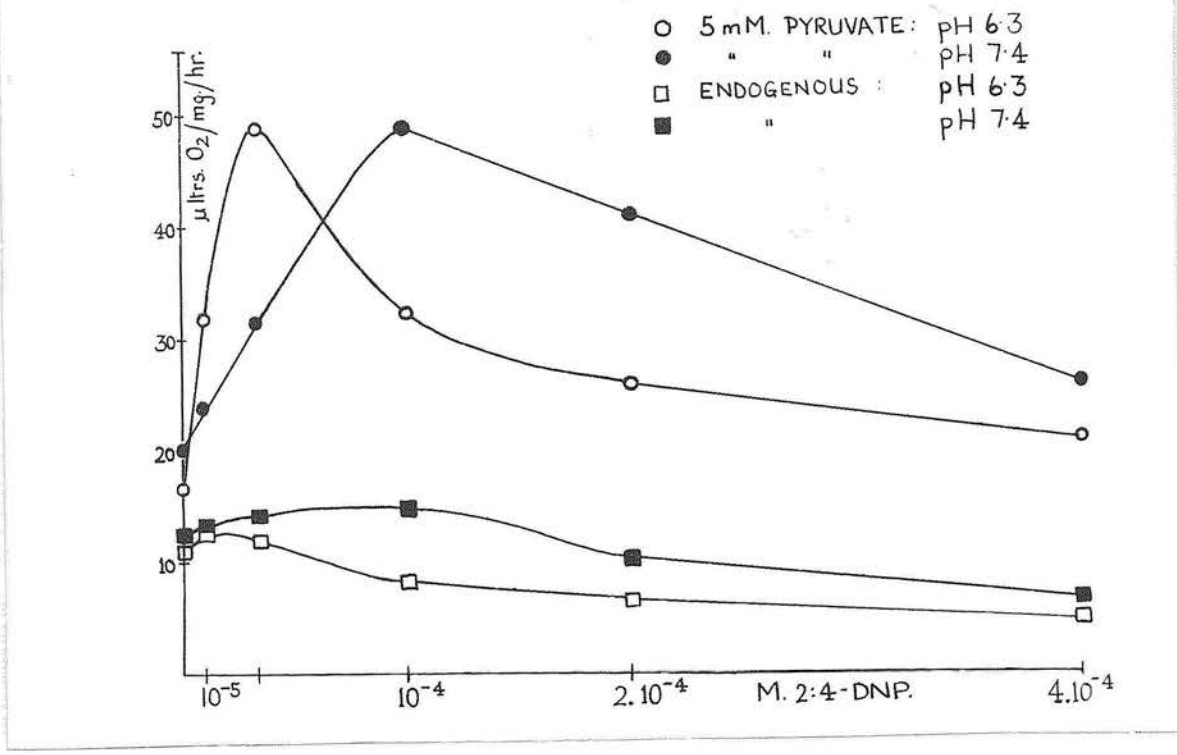


GRAPH 35

EFFECT OF pH ON SUCCINATE RESPIRATION.


△ 40 mM. SUCCINATE : pH 6·3
▲ " " " pH 7·4
□ ENDOGENOUS pH 6·3
■ " pH 7·4
µ ltrs. O2/mg/hr.
40
30
20
10
10-5
10-4
2.10-4
M. 2:4-DNP.
4.10-4

GRAPH 36


5 mM. PYRUVATE: pH 6·3
" " pH 7·4
ENDOGENOUS : pH 6·3
" pH 7·4
µ ltrs. O2/mg./hr.
50
40
30
20
10
10-5
10-4
2.10-4
M. 2:4-DNP.
4.10-4

GRAPH 38

D I S C U S S I O N

The foregoing experiments indicate that succinate at pH 7.4 does not readily enter intact L cells. In this respect Giebels and Passou (1960) concluded that the exchange rate for succinate across the erythrocyte membrane was upwards of 225 times slower than for the monocarboxylic acids. Although succinate has been cited by many authors as an efficient exogenous substrate for respiration, it would appear that in many cases the concentrations of succinate used have been much greater than the usual 5 - 10mM range. Kratzing (1953) examining the metabolism of guinea pig cerebral slices found that 20mM succinate was required compared with only 5mM glucose. Woodford (1956) similarly used 22mM succinate in his work on minced rat brain, and Planterose (1961) 50mM on pig kidney cells. In some cases, succinate has been used at high concentrations with little regard for the tonicity of the medium. For example, it has been reported that sodium succinate considerably stimulates the respiration rate of L cells (Phillips and Terryberry 1957). The concentration used by these authors was 200mM which must have caused severe osmotic shock and membrane damage considering that isotonicity is equivalent to about 156mM sodium chloride, and the medium was buffered besides. In some cases where smaller concentrations of succinate have produced considerable changes in respiration, it is obvious that the work has been done on rapidly dying systems with membranes unlikely to be in good condition - for example, that of Webb, Saunders and Thienes (1949) on rat heart slices, and Johnson and Johnson (1962) on L cells. More direct evidence for the state of the plasma membrane governing to a large extent the respiration rate of succinate comes from the work of Koefoed-Johnsen and Mann (1954) who found that the oxidation rate of succinate by thick suspension of ram sperm was greatly increased on prolonged storage of the sperm and on treatment by surface agents.

The results of the foregoing experiments involving 2:4-DNP and pH confirm succinate to be an unnatural exogenous substrate with no specific transport mechanism, and with an ionic equilibrium at pH 7.4 which renders it essentially indifferent to the physiological membrane of the L cell - a useful feature for an important intermediary of oxidative metabolism.

At pH 7.4 the direct relationship between respiration rate and exogenous succinate concentration up to 42.5mM, indicates that over this concentration range respiration rate is limited by the availability of succinate. Furthermore, uncoupling of oxidative phosphorylation by concentrations of 2:4-DNP between 0.01 and 0.40mM has little or no effect in increasing the respiration of 40mM. succinate, although as has been shown elsewhere, the respiration rates of glucose, lactate and pyruvate are all substantially increased on uncoupling. This further supports the idea that even with external concentrations of 40mM the rate of succinate entry at pH 7.4 is low and limits respiration rate.

As Overton (1895), Danielli (1953), Hober (1945) and others have pointed out, the penetration of the predominantly hydrophobic osmotic barrier of most physiological membranes by simple diffusion, is greatly influenced by the degree of hydration of the ion or solute. The pK values for succinate (4.18 and 5.55) indicate that at pH 7.4 the ratio of double charged ions to single charged ions to undissociated molecules is $1:1.43 \times 10^{-2} : 8.5 \times 10^{-6}$. At an external succinate concentration of 42.5mM therefore, undissociated molecules which might be expected to penetrate the plasma membrane readily across the lipoid phase are present in very low concentrations (0.36×10^{-5} mM) and single charged ions present at only 0.59mM concentration. A decrease in pH from 7.4 to 6.3 is accompanied by an increase in the concentration of undissociated molecules to 0.05mM and single charged ions to 6.4mM.

At pH 6.3 respiration of succinate in the absence of 2:4-DNP is rather lower than at pH 7.4, although the above considerations indicate that much more substrate is present which can permeate the membrane. The most plausible

explanation for this is that whereas at pH 7.4 substrate is limiting, at pH 6.3 the pH exerts an inhibitory effect on respiratory metabolism. The extent to which 2:4-DNP stimulates respiration at pH 6.3 must be interpreted as evidence that much more substrate is available to the oxidation system than at pH 7.4. The ability of uncouplers to stimulate respiration at pH values so low that respiration in the absence of uncouplers is slightly depressed suggests that in the latter case, the depressive effect of low pH is possibly on some aspect of respiratory chain phosphorylative metabolism which is bypassed on uncoupling.

The possibility that the increased rate of succinate entry at pH 6.3 is due to damage to the plasma membrane at low pH rather than to increase in undissociated molecules and single charged ions is thought unlikely for the following reasons:- An effect of low pH on membrane integrity would most likely increase with time. The respiration rates with succinate at low pH however were constant with time for up to 2 hours. Furthermore, subjection to pH 6.3 for an hour did not significantly increase the sensitivity of cells to dye.

The results therefore provide good circumstantial evidence that by moving the equilibrium for the dissociation of succinic acid in the direction of the undissociated molecule by decreasing pH within the physiological range, a significant increase in the efficiency of entry can be achieved. It would appear that the fully ionised form cannot easily penetrate intact plasma membranes, and that entry is achieved by the free acid and the single charged ion, both of which are greatly increased by decreasing the pH to 6.3. A precedent for this conclusion comes from the work of Barron, Ardao and Hearon (1950) who showed that a lowering of pH from 7.0 to 5.3 induced a four-fold increase in the respiration rate for succinate (10mM) by Mycobacterium creativorans. Mitchell and Moyle have also observed that entry of various organic acids into micro organisms by non-specific mechanisms occurs more easily at low pH values.

The work on L cells described in this paper appears to be the only investigation on the effect of pH on succinate entry into mammalian cells.

BIBLIOGRAPHY.

1. Abraham, S., Hirsch, P.F. and Choikoff, I.L. (1954) J. Bid. Chem. 211, 31.

2. Adenobonojo, F.O., Bensch,K.G. and King,D.W. (1961) Cancer Res. 21 252

3. Aebi, H. (1952) Biochim Biophys. Acta 9, 443.

4. Ambe, K.S. and Venkataraman, A. (1959) Biochem.Biophys. Res.Comm,1, 133.

5. Ansell, B.M. (1963) Salicylates, Empire Rheumatism Council. edit. by A.St.J. Dixon, B.K. Martinetal. Churchill, 1963.

6. Ashmore, J., Cahill, G.F.Jnr. and Hastings A.B. (1960) Recent Progress in Hormone Research 16, 547.

7. Bailey, J.M., Gey G.O. and Gey M.K. (1959) J. Biol.Chem.234. 1042.

8. Balazs, R, Magyar. K. and Richter, D. (1964) Proc. 5th Int. Neurochemistry Symp. Pergamon Press, London.

9. Barker, S.B. and Summerson, W.H. (1941) J. Biol. Chem. 138, 535.

10. Barnett, H.L., Powers, J.R., Benward. J.H. and Hartmann, A.F. (1942) J. Pediat, 21, 214.

11. Barron, E.S.G. and Harrop, G.A.Jnr. (1928) J. Biol. Chem.79, 651.

12. Beevers, H. and French. R.C. (1953) Biol.Revs. 28, p.460.

13. Belitzer, V.A. and Tsibakowa, E.T. (1939) Biokhimiya 4, 516.

14. Beloff-Chain, A., Catanzaro, R., Chain, E.B., Longinoffi, L., Masi, I and Pocchiari, F. (1962). Biochim. Biophys. Acta. 56, 153.

15. Blackman, G.E., Templeman. W.G. and Halliday, D.J. (1951) Ann.Rev. Plant Physiol. 2, 199.

16. Bland, D.M. and Whittam. R. (1964) Biochem.J. 92, 158.

17. Bodine, J.H. (1926) Science 64, 532.

18. Bollet, A.J. (1961) Athritis and Rheumatism 4, 624.

19. Bonner, W.D. (1951) Biochem.J. 49, VIII.

20. Bonner, W.D. (1954) Biochem.J. 56, 274.

21. Borst, P, and Slater, E.C. (1961) Biochim. Biophys. Acta 48, 362.

22. Boyer, P.D. (1958) Proc.Int.Symp.Enz.Chem. p.301, Tokyo-Kyato, Tokyo.

23. Boyer, P.D., De Luca, M., Ebner, K.E., Hultquist, D.E. and Peter, J.B. (1962) J. Biol. Chem. 237, PC.3306.

24. Brierley, G.P., Hura, E. and Green, D.E. (1963) Science 140, 60.

25. Brody, T.M. (1956) J. Pharm. Exp. Therap. 117, 39.

26. Brown, A.W.A. (1951) Insect Control by Chemicals, John Wiley, N.York.

27. Bryant, J.C., Evans, V.J., Scilling, E.L. and Earle, W.R. (1961) J. Nat. Cancer Inst. 26, 239.

28. Bryant, J.C., Scilling, E.L., Evans, V.J. and Earle, W.R. (1960) J. Nat. Cancer Inst. 24, 859.

29. Bryant, C., Smith, M.J.H. And Hines, W.J.W. (1963) Biochem. J. 86, 391.

30. Burton, K. (1956) Biochem. J. 62, 315.

31. Cailleau, R., Moss, S. and Siegal. B.V. (1956) J. Nat. Canc. Inst. 16, 1011.

32. Caldwell, P.C. (1954) J. Physiol. 126, 169.

33. Caldwell, P.C. (1956) Ann.Rev.Cyt. 5, 229.

34. Caldwell, P.C. (1958) J. Physiol. (Lond.) 142, 22.

35. Carrel, A. and Ebeling, A.H. (1923) J. Exp. Med. 38, 419.

36. Carroll, N.V., Longley, R.W. and Roe, J.H. (1956) J. Biol.Chem.220, 583.

37. Cavallini, D., Frontali, W. and Toschi, G. (1949) Nature 164, 792.

38. Cazeneuze, P. and Lepine, R. (1885) Compt. Rend. 101, 1167.

39. Ceriotti, G. (1955) J. Bid. Chem. 214, 59.

40. Chambers, R. and Chambers, E.L. (1961) Explorations into the Nature Of the Living Cell, Harvard University Press.

41. Chambers, R., Pollack, H. and Hiller, S. (1927) Proc. Soc. Exp. Biol. Med. 24, 760.

42. Chan. P.C., Lehninger, A.L. and Enns, T. (1960) J. Biol. Chem. 235, 1790.

43. Chance, B. (1956) in O.H. Gaebler, Enzymes, Units in Biological Structure and Function. p.447. Acad. Press. N.York.

44. Chance, B. (1959) Ciba Foundation Symposium on the Regulation of Cell Metabolism. p.91. Churchill, 1959.

45. Chance, B. (1961) Ciba Foundation Symposium on Quinones, p.327. Churchill, 1961.

46. Chance, B. and Conrad, H. (1959) J. Biol. Chem. 234, 1568.

47. Chance, B. and Hess., B. (1956) Ann. N.Y. Acad. Sc.63, 1008.

48. Chance, B. and Hollunger, G. (1957) Fed. Proc. 16, 163.

49. Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 429.

50. Chance, B. and Williams, G.R. (1956) Adv. Enzymol 17, 65.

51. Chang, R.S. and Geyer, R.P. (1957) Proc. Soc. Exp. Biol.Med. 96, 336.

52. Chappell, J.B., Greville, G.D. and Bicknell, K.E. (1962) Biochem. J. 8, 61P.

53. Chappell, J.B. and Greville, G.D. (1960) Biochim. Biophys. Acta. 38, 483.

54. Charnock J.S. and Opit, L.J. (1962) Biochem. J. 83, 602.

55. Christensen, H.N., Riggs, T.R., Fisher, H. and Palatine, I.M. (1952) J. Biol. Chem. 198, 1.

56. Christensen, H.N. Riggs and Coyne, B.A. (1954) J. Biol. Chem. 209, 413.

57. Christensen, H.N. (1960) Adv. Protein Chem. 15, 239.

58. Clarke, B.M. and Todd, A. (1961) Ciba Foundation Symposium on Quinones p.190, Churchill, 1961.

59. Clifton, C.E. and Logan, W.A. (1939) J. Bact. 37, 523.

60. Clowes, G.H.A., and Keltch, A.K. (1951) Proc. Soc. Exp. Biol. Med. 77, 369.

61. Clowes, G.H.A. and Keltch, A.K. (1954) Proc. Soc. Exp. Biol. Med. 86, 629,

62. Cohn, M. and Drysdale, G.R. (1956) J. Biol. Chem. 216, 831.

63. Cohn, M. and Drysdale, G.R. (1958) J. Biol. Chem. 223, 1574.

64. Cooper, C. and Lehninger, A.L. (1956) J. Biol. Chem. 219, 489.

65. Cooper, C. and Lehninger, A.L. (1957) J. Biol. Chem. 224, 561.

66. Cooper, P.D., Wilson, J.N. and Burt, A.M. (1959) J. Gen. Microbiol. 21, 702

67. Crane, F.L., Hatefi, Y., Lester, K.L. and Widmer, C. (1957) Biochim. Biophys. Acta 25, 220.

68. Cremona, T. and Kearney, E.B. (1964) J. Biol. Chem. 239, 2328.

69. Crockett, R.L. and Leslie, I. (1963) Biochem. J. 89, 516.

70. Dales, S. (1960) Canad. J. Biochem. Physiol. 38, 871.

71. Dales, S. and Fisher, K.C. (1959) Canad. J. Biochem. Physiol. 37, 623.

72. Danes, B.S. (1957) Exp. Cell. Res. 12, 169.

73. Danes, B.S. and Paul, J. (1961) Exp. Cell. Res. 24, 344.

74. Danielli, J.F. (1953) Cytology and Cell Physiol. edit. G. Bourne. Oxford UP

75. Dawkins, M.J.R., Judah, G.D. and Rees, K.R. (1959) Biochem. J. 73, 16.

76. De Luca, H.F. and Engstrom, G.W. (1961) Proc. Nat. Acad. Sc. U.S. 47, 1744.

77. Dewan, J.G. and Green, D.E. (1938) Biochem. J. 32, 626, 1200.

78. Dodds, E.C. and Greville, G.D. (1934) Lancet, 226, 398.

79. Dreywood, R. (1946) Ind. Eng. Chem. (Anal. Edit.) 18, 499.

80. Dumont, J.E. (1956) Biochim. Biophys. Acta. 56, 382.

81. Eagle, H. (1955) J. Bid. Chem. 214, 839.

82. Eagle, H., Barban, S., Levy, M. and Schulze, H.D. (1958) J. Bid. Chem. 233, 551.

83. Eagle, H. Oyama, V.I., Levy, M., Horton, C.L. and Fleischman, R. (1956) J. Biol. Chem. 218, 607.

84. Earle, W.R. (1943) J. Nat. Canc. Inst. 3, 555.

85. Earle, W.R., Bryant, J.C., Schilling, E.L. and Evans, V.J. (1956) Ann. N.Y. Acad. Sc. 63, 666.

86. Edelhoch, H. Haysishi, O. and Teply, L.J. (1952) J. Biol. Chem. 197, 97.

87. Ehrenfest, E. and Ronzani, E. (1933) Proc. Soc. Exp. Biol. Med. 31, 318.

88. Ehrenfest, E. and Ronzani, E. (1935) Proc. Soc. Exp. Biol. Med. 34, 8.

89. Ellis, D.B. and Scholefield, P.G. (1961) Cancer Res. 21, 650.

90. El Hawary, M.F.S. and Thompson, R.H.S. (1953) Biochem. J. 53, 340.

91. Emmelot, P. and Bos, C.J. (1959) Brit. J. Canc. 13, 520.

92. Emmelot, P. and Nout, S.J. (1959) Brit. J. Canc. 13, 513.

93. Erganian, J.A., Forbes, G.B. and Case, D.M. (1947) J. Pediat. 30, 129.

94. Ernster, L. (1960) IUB/IUBS. Symp. on Biological Structure and Function, Vol. 2, p.139, Acad. Press. N.Y.

95. Ernster, L. (1961) Proc. V. Int. Congress. Biochem. (Moscow) Vol.5, 115. Pergamon Press. 1963.

96. Evans, V.J., Bryant, J.C. McQuilkin, W.T., Fioramonti, M.C. Sanford, K.K., Westfall, B.B. and Earle, W.R. (1956) Cancer Res. 16, 87.

97. Faed, M. (1955) B.Sc. Thesis, University of Edinburgh.

98. Fioramonti, M.C., Evans, V.J. and Earle, W.R. (1958) J. Nat. Canc. Dist. 21, 579.

99. Fisher, R.A. (1948) Statistical Methods for Research Workers (10th edit) Oliver and Boyd, Edinburgh.

100. Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375.

101. Friedkin, M. and Lehninger, A.L. (1949) J. Biol. Chem. 178, 611.

102. Friedemann, T.E. and Haugen, G.E. (1943) J. Biol. Chem. 147, 415.

103. Fulton, W.C., Sinclair, R. and Leslie, I. (1956) Biochem. J. 63, 18P.

104. Gatt, S., Krimsby, I. and Racker, E. (1956) Fed. Proc. 15, 259.

105. Giebbels, O. and Passou, H. (1960) Pflugers Arch. 271, 378.

106. Gifford, G.E., Robertson, H.E. and Syverton, J.T. (1956) Anat. Record 124, 487.

107. Graff, S. and McCartney, K.S. (1957) Exp. Cell. Res. 13, 348.

108. Green, D.E. (1959) Adv. Enzymol 21, 73.

109. Green, D.E. (1961) Ciba Foundation Symp. on Quinones p.130. Churchill.

110. Green, D.E. (1964) Proc. VI. Int. Congress Biochem. N.Y. vol.8, 615.

111. Greville, D.G. (1959)

112. Greville, D.G. (1962) in Discussion Biochem. Soc. Symp. on structure and function of the membranes and surfaces of cells, London.

113. Haas, E., Harrer, C.J. and Hogness, T.R. (1942) J. Biol. Chem. 143, 341.

114. Harden, A. and Young, W.J. (1906) Proc. Roy. Soc. London B. 77, 405.

115. Harris, H. (1956) Brit. J. Exp. Path. 37, 512.

116. Harris, M. and Kutsky, P.B. (1953) J. Cell. Comp. Physiol, 42, 449.

117. Hatefi, Y, Haavik, A.G., Fowler, L.R. and Griffiths, D.E. (1962) J. Biol. Chem. 237, 2661.

118. Healey, G.M. (1955) Proc.Soc.Exp. Biol. Med. 89, 71

119. Healy, G.M., Fisher, D.S. and Parker, R.C. (1954) Canad. J. Biochem. Physiol. 32, 319.

120. Heinz, E. and Walsh, P.O. (1958) J. Biol. Chem. 233, 1488.

121. Henker, H.C. (1962) Biochim. Biophys. Acta 60, 646.

122. Henker, H.C. (1962) Biochim. Biophys. Acta 63, 46.

123. Henker, H.C. (1963) Biochim. Biophys. Acta 73, 311.

124. Henker, H.C. (1964) Biochim. Biophys. Acta 81, 1.

125. Hess, B. (1961) Proc. V. Int. Congress Biochem. Moscow. vol. 5, 313.

126. Heymans, C. and Bouchaert, J.J. (1928) Arch. Int. Pharmacodyn 35, 63.

127. Hicklin, J.A. (1959) Nature 184, 2029.

128. Hill, A.V. (1955) Proc. Roy. Soc. Lond. B. 144, 1.

129. Hill, R.B., Bensch, K.G., Simbonis, J. and King, D.W. (1959) Nature 183, 1018.

130. Hober, R. (1945) Physical Chemistry of Cells and Tissues. J. and A. Churchill, London.

131. Horecker, B.L. (1950) J. Biol. Chem. 183, 593.

132. Horecker, B.L. and Mehler, A.H. (1955) Ann. Rev. Biochem. 24, 207.

133. Hotchkiss, R.D. (1946) in D.E. Green. Currents in Biochemical Research p.379, Inter-science, N.York.

134. Howland, J.L. (1963) Biochim. Biophys. Acta 77, 419.

135. Huennekens, F.M., Felton, S.P., Rao, N.A. and Mackler, B (1961) Fed. Proc. 20, 45.

136. Huggins, A.K., Moses, V and Smith, M.J.H. (1960) Biochem. J. 76, 11P.

137. Huggins, A.K., Smith, M.J.H. and Moses, V. (1961) Biochem. J. 79, 271.

138. Huggins, A.K. Bryant, C. and Smith, M.J.H. (1961) J. Pharm. Pharmacol. 13, 654.

139. Hullin, R.P. and Noble, R.L. (1953) Biochem. J. 55, 289.

140. Hullsman, W.C., Elliot, W.B. and Slater, E.C. (1960) Biochim. Biophys. Acta. 39. 267.

141. Hunter, F.E. (1949) J. Biol. Chem. 177, 361.

142. Hunter, F.E. (1951) Phosphorus Metabolism. vol.1 John Hopkins Press.

143. Hunter, F.E. (1961) Proc. V. Int. Congress Biochem. Moscow. vol.5, 287.

144. Hurlbert, R.B. Schmitz, H.S., Brumm, A.F. and Potter, V.R. (1954) J. Biol. Chem. 209, 23.

145. Hutchison, W.C. and Munro, H.N. (1961) The Analyst 86, 768.

146. Ibsen, K.H., Coe, E.L. and McKee, R.W. (1958) Biochim. Biophys. Acta 30, 384.

147. Jeffrey, S.W. and Smith, H.J.H. (1959) Biochem. J.72, 462.

148. Johnson, M.K. and Johnson, E.J. (1962) Proc. Soc. Exp. Biol. Med. 111,149.

149. Johnston, R.M. (1959) Canad. J. Biochem. Physiol. 37, 589.

150. Jones, M. and Bonting, S.L. (1956) Exp. Cell. Res. 10, 631.

151. Jones, V.C., Morris, J.L. and Landon, E.J. (1963) Biochim. Biophys. Acta 71, 462.

152. Katsuta, H., Takaoka, T., Mitamura, K., Kawada, I., Kawabara, H. and Kuwabara, S. (1959) Jap. J. Exp. Med. 29, 191.

153. Judah, J.D., (1951) Biochem. J. 49, 271.

154. Katsuta, H. and Takaoka, T. (1960) Jap. J. Exp. Med. 30, 235.

155. Kaplan, E.H., Kennedy, J. and Davis, J. (1954) Archiv. Biochem. 51, 47.

156. Keilin, D. (1925) Proc. Roy. Soc. Lond. B.98, 312.

157. Keilin, D. and Hartree, E.F. (1939) Proc. Roy. Soc. Lond. B. 127, 167.

158. Kennedy, E.P. and Lehninger, A.L. (1948) J. Biol. Chem. 172, 847.

159. Kennedy, E.P. and Lehninger, A.L. (1949) J. Biol. Chem. 179, 957.

160. Klingenberg, M. (1964) Proc. VI Int. Cong. Biochem. N.York. vol.9, 699.

161. Klingenberg, M. and Bucher, T. (1960) Ann. Rev. Biochem. 29, 669.

162. Kielley, W.W. (1955) Methods in Enzymology, Vol.II, p.593. edit. S.P. Colowick and N.O. Kaplan.

163. Kielley, W.W. and Kielley, R.K. (1951) J. Biol. Chem. 191, 485.

164. King, T.E. and Howard, R.L. (1960) Biochim. Biophys. Acta. 12, 33.

165. King, T.E. and Howard, R.L. (1962) Biochim. Biophys. Acta. 59, 489.

166. King, T.E. Howard R.L. and Wilson, D. (1961) Proc. V. Int. Cong. Biochem., Moscow. vol.5, 193. Pergamon Press, Lond.1963.

167. Kirpekar, S.M. and Lewis, G.G. (1960) Brit. J. Pharmacol. 15, 175.

168. Kitos, P.A., Sinclair, R. and Waymouth, C. (1962) Exp. Cell. Res. 27, 307.

169. Koefoed-Johnsen, H.H. and Mann, T. (1954) Biochem. J. 57, 406.

170. Kornberg, A. (1955) Methods in Enzymology, vol.1. p.441. edit. S.P. Colowick and N.O. Kaplan.

171. Kotsyuk, P.G. and Sorokina, B.A. (1961) in Membrane Transport and Metabolism, edit. A. Kleinzeller and A. Kotyk, Academic Press.

172. Krahl, M.E. (1950) Biol. Bull. Woods Hole 98, 175.

173. Krahl, M.E. Meltch, A.K. and Clowes, G.H.A. (1940) J. Biol.Chem.136, 563.

174. Kratzing, C.C. (1953) Biochem. J. 54, 312.

175. Krebs, H.A. (1937) Biochem. J. 31, 661.

176. Krebs, H.A., and Johnson, W.A. (1937) Biochem. J. 31, 772.

177. Krebs, H.A. and Homs, R. (1953) Biochim. Biophys. Acta 12, 172.

178. Krimsby, I. and Racker, E. (1955) Science 122, 319.

179. Kulka, R.G. and Cooper, C. (1962) J. Biol. Chem. 237. 936.

180. Lardy, H.A. and Elvehjam, C.A. (1945) Ann. Rev. Biochem. 14, 1.

181. Lardy, H.A. and McMurray, W.C. (1959) Fed. Proc. 18, 269.

182. Lardy, H.A. and Wellman, H. (1953) J. Biol. Chem. 201, 357.

183. Lardy, H.A. and Phillips, P.H. (1943) J. Biol. Chem. 149, 177.

184. Lardy, H.A. and Phillips, P.H. (1945) Archiv. Biochem. 6, 53.

185. Lehninger, A.L. (1949) J. Biol. Chem. 178, 625.

186. Lehninger, A.L. (1951) Phosphorus Metabolism. Vol.1 John Hopkins Press.

187. Lehninger, A.L. (1953) Harvey Lectures. Serv.49, 176.

188. Lehninger, A.L. (1959) J. Biol. Chem. 234, 2187.

189. Lehninger, A.L. (1961) Proc. V. Int. Congress Biochem. Moscow p.239.

190. Lehninger, A.L. and Wadkins, C.L. (1962) Ann. Rev. Biochem. 31, 47.

191. Lemberg, P. (1961) Adv. Enzymol. 23, 365.

192. Leslie, I. Fulton, W.C. and Sinclair, R. (1957) Biochim. Biophys. Acta 24, 365.

193. Lieberman, I. and Ove, P. (1958) J. Biol. Chem. 233, 634.

194. Lipmann, F. (1939) Nature 143, 281.

195. Loomis, W.F. and Lipmann, F. (1948) J. Biol. Chem. 173, 807.

196. Low, H., (1959) Biochim. Biophys. Acta 32, 1.

197. Low, H., Siekevitz, P., Ernster, L. and Lindberg, O. (1958) Biochim. Biophys. Acta, 29, 392.

198. Lowry, O.H. (1962) Biochem. Biophys. Res. Comm. 7, 10.

199. Lowry, O.H. Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265.

200. Lynen, F. (1941) Liebigs Ann. 546, 120.

201. Lynen, F (1958) in Neure Ergebnisse Chemie u Stoffwechsel der Kohlenhydrate, Berlin. Springer Verlog. p.155.

202. Lynen, F., Hartmann, G., Netter, K.F. and Schuegraf, A. (1959) Ciba Foundation. Symposium on the Control of Cell Metabolism p.256. Churchill, Lond.

203. McIlwain, H. and Gore, M.B.R. (1951) Biochem. J. 50, 24.

204. McLimmons, W.F., Davis, E.V. Glover, F.L. and Rake, G.W. (1957) J. Immun. 79, 428.

205. McQuilkin, W.T., Evans, V.J. and Earle, W.R. (1957) J. Nat. Cancer Inst. 19, 885.

206. Mahler, H.R., Sarkar, N.K., Vernon, R.P. and Alberty, R.A. (1952) J. Biol. Chem. 199, 585.

207. Maic, J.J. and Rickenberg, H.V. (1962) Exp. Cell. Research, 27, 31.

208. Manchester, K.L., Randle, P.J. and Smith, G.H. (1958) Brit. Med. J. 1, 1028.

209. Mansour, T.E. and Mansour, J.M. (1962) J. Biol. Chem. 237, 629.

210. Marks, V., Smith, M.J.H. and Cunliffe, A.C.J. (1961) J. Pharm. Pharmacology, 13, 218.

211. Martin, A.W. and Field, J. (1934) Proc. Soc. Exp. Biol. Med. 32, 54.

212. Martius, G. and Nitz-Litzow, M. (1953) Biochim. Biophys. Acta 12, 134.

213. Massey, V. (1958) Biochim. Biophs. Acta. 30, 205.

214. Medes G. and Weinhouse, S. (1958) Cancer Res. 18, 352.

215. Mehler, A.H., (1948) J. Biol. Chem. 174, 961.

216. Melrose, D.R. and Terner, C. (1953) Biochem. J. 53, 296.

217. Merola, A.J., Coleman, R. and Hansen, R. (1963) Biochim. Biophys. Acta. 73, 638.

218. Meyerhoff, O. (1949) J. Biol. Chem. 180, 575.

219. Mitchell, P. (1961) Nature 191, 144.

220. Mitchell, P. (1961b) Biochem. J. 79, 23P.

221. Mohberg, J. and Johnson, M.J. (1963) J. Nat. Cancer Inst. 31, 611.

222. Monod, J. (1944) Ann. Inst. Pasteur 70, 381.

223. Morrison, M. and Horie, S. (1964) J. Biol. Chem. 239, 1438.

224. Moses, V. and Syrett, P.J. (1955) J. Bact. 70, 201.

225. Mudge, G.H. (1951) Amer, J. Physiol. 167, 206.

226. Munyon, W.H. and Merchant, D.J. (1959) Exp. Cell. Res. 17, 490.

227. Myers, D.K. and Slater, E.C. (1957) Biochem. J. 67, 558, 572.

228. Newsholme, E.A. and Randle, P.J. (1961) Biochem. J. 80, 655.

229. Newsholme, E.A. and Randle, P.J. and Manchester, K.L. (1962) Nature 193, 270

230. Nicholls, P. (1963) Biochim. Biophys. Acta 73, 667.

231. Nielson, S.O. and Lehninger, A.L. (1954) J. Amer. Chem. Soc. 76, 3860.

232. Novikoff, A.B. (1961) in 'The Cell' Vol.II. p.299. edit. Brachet, Mirsky, Acad. Press, N.York.

233. Ochoa, S. (1940) Nature 146, 267.

234. Ochoa, S. (1941)

235. Ochoa, S. (1943) J. Biol. Chem. 151, 493.

236. Ochoa, S. (1955) Methods in Enzymology. Vol.I. p.735. edit. S.P. Colowick and N.O. Kaplan. Acad. Press. N.York.

237. Overton, E. (1895) Vjschr, naturf. Ges. Zurich 40, 159.

238. Packer, L. (1960) J. Biol Chem. 235, 242.

239. Pasieka, A.E. Morton, H.J. and Morgan, J.F. (1958) Canad. J. Biochem. Physiol. 36, 771.

240 Paul, J. (1959) J. Exp. Zool, 142, 475.

241. Peiss, C.N. and Field, G. (1952) Arch. Biochem. 36, 276.

242. Peter, G.B. and Boyer, P.D. (1963) J. Biol. Chem. 238, PC.1180.

243. Phillips, H.J. and Andrews, R.V. (1960) Proc. Soc. Exp. Biol. Med. 103, 160.

244. Phillips, H.J., Andrews, R.V. and Skank, V. (1960) Exp. Cell Res. 20, 607.

245. Phillips, H.J. and Feldhaus, R.J. (1956) Proc. Soc. Exp. Biol. Med. 92, 478.

246. Phillips, H.J. and Terryberry, J.E. (1957) Exp. Cell. Res. 13, 341.

247. Phillips, H.J. and Terryberry, J.E. (1958) Exp. Cell. Res. 14, 454.

248. Pickett, M.J. and Clifton, C.E. (1943) J. Cell. Comp. Physiol. 22, 147.

249. Pinchot, G.B. (1963) Fed. Proc. 22, 1076.

250. Pollack, H. (1928) Biol. Bull. 55, 383.

251. Pomerat, C.M. and Willmer, E.N. (1939) J. Exp. Biol. 16, 232.

252. Pressman, B.C. and Lardy, H.A. (1952) J. Biol. Chem. 197, 547.

253. Pressman, B.C. and Lardy, H.A. (1955) Biochim. Biophys. Acta 18, 482.

254. Pressman, B.P. and Dallam, R.D. (1961) Proc. V. Int. Cong. Biochem. Moscow p.470, Pergamon Press.

255. Purvis, J.L. (1958) Nature 182, 711.

256. Purvis, J.L. (1960) Biochim. Biophys. Acta 38, 435.

257. Rabinovitz, M., Olsen, E. and Greenberg, D.M. (1955) J. Biol. Chem. 213, 1.

258. Racker, E. (1956) Ann. N.Y. Acad. Sc. 63, 1017.

259. Racker, E. and Wu. R. (1959) Ciba Foundation Symposium on the Regulation of Cell Metabolism. p.205 Churchill, London.

260. Ram, D., Kalner, H.S. and Bloch-Frankenthal, L. (1963) Cancer Res. 23, 600.

261. Ramirez, J. (1954) J. Physiol. (London) 147, 44.

262. Ramirez, J. and Mujica, A. (1961) Fed. Proc. 20, 26.

263. Ramirez, J. and Mujica, A. (1964) Biochim. Biophys. Acta 86, 1.

264. Randle, P.J. and Smith, G.H. (1958) Biochem. J. 70, 490.

265. Redfearn, E.R. (1960) Annual Report Chem. Soc. 57, 395.

266. Redfearn, E.R. (1961) Ciba Foundation Symposium on Quinones p.346, Churchill, London.

267. Reid, R.A. (1960) B. Sc. Thesis. University of Edinburgh.

268. Reznikoff, P. and Pollack, H. (1928) Biol. Bull. 55, 377.

269. Riggs, T.R., Walker, L.M. and Christensen, H.N. (1958) J. Biol. Chem. 233, 1479.

270. Riggs, T.R., Walker, L.M. (1963) J. Biol. Chem. 238, 2663.

271. Ringler, R.L., Minakami, S. and Singer, T.P. (1960) Biochem. Biophys. Res. Comm. 3, 417.

272. Rous, P. (1925) J. Exp. Med. 41, 399.

273. Salomon, L.L. and Johnson, J.E. (1959) Arch. Biochem. Biophys. 82, 179.

274. Sanford, K.K., Earle, W.R. and Likely, G.D. (1948) J. Nat. Cancer. Inst. 9, 229.

275. Sanford, K.K., Likely, G.D. and Earle, W.R. (1954) J. Nat. Cancer. Inst. 15, 215.

276. Schlenk, F. and Von. Euler, H. (1936) Naturwissenschaften. 24, 794.

277. Schmid, W. (1957) Naunym-schmiedeberg's Arch.Exp.Path u Pharmakol. 232, 351.

278. Schneider, W.C. (1957) in Methods in Enzymology, vol.III. p.680. edit. S.P. Colowick and N.O. Kaplan. Acad. Press.

279. Schneider, W.C. and Hogeboom, G.H. (1950) J. Biol. Chem. 183, 123.

280. Selvaraj, R.J. and Sbarra, A.J. (1964) Proc. VI. Int. Cong. Biochem. N.York. Abstract VI, 107.

281. Shacter, B. (1955) Arch. Biochem. Biophys. 57, 387.

282. Siekevitz, P. and Potter, V.T. (1953) J. Biol. Chem. 201, 1.

283. Simon, E.W. (1953) Biol. Revs. 28, 453.

284. Simon, E.W. and Beevers, H. (1952) New Phytol. 51, 163.

285. Siminovitch, I., Graham, A.F., Lesly, S.M. and Nevill, A. (1957) Exp. Cell. Res. 12, 299.

286. Sinclair, R. (1958) Ph.D. Thesis. Queens University of Belfast.

287. Sinclair, R. (1964) Unpublished.

288. Sinclair, R., Reid, R.A. and Mitchell, P. (1963) Nature 197, 982.

289. Singer, T.P., Minakami, S. and Ringler, R.L. (1961) Proc. V. Int. Cong. Biochem. Moscow. vol.5, 174. Pergamon Press, 1963.

290. Slater, E.C. (1953) Nature 172, 975.

291. Slater, E.C. (1955) Proc. III Int. Cong. Biochem. Brussels, p.264. Acad. Press, 1956.

292. Slater, E.C. (1958) Revs. Pure App. Chem. (Australia) 8, 221.

293. Slater, E.C. (1958b) Adv. Enzymol 20, 147.

294. Slater, E.C. (1958c) Biochem. Soc. Symposium.

295. Slater, E.C. (1961) Proc. V. Int. Cong. Biochem. Moscow. vol.5, 325.

296. Slater, E.C. (1964) Oxidative Phosphorylation (in press)

297. Slater, E.C., Colpa-Boonstra, J.P. and Links, J. (1961) Ciba Foundation Symposium on Quinones p.161 Churchill, London.

298. Slater, E.C. and Hulsman, W.C. (1959) Ciba Foundation Symposium on the Regulation of Cell Metabolism, p.58, Churchill, London.

299. Slater, E.C., Kemp, T. and Tager, J.M. (in press)

300. Slater, E.C. and Lewis, S.E. (1954) Biochem. J. 58, 337.

301. Sibley, J.A. and Lehninger, A.L. (1949) J. Biol. Chem. 177, 859.

302. Smith, A.L. and Lester, A.L. (1960) Fed. Proc. 19, 34.

303. Smith, M.J.H. (1959) J. Pharm. Pharmacol. 11, 705.

304. Smith, M.J.H. (1963) Empire Rheumatism Council Symposium on Salicylates. edit. by A.St.J. Dixon, B.K. Martin et al. Churchill, 1963.

305. Smith, M.J.H. and Moses, V. (1960) Biochem. J. 74, 17P.

306. Snoswell, A.M. (1961) Biochim. Biophys. Acta 52, 216.

307. Snoswell, A.M. (1962) Biochim. Biophys. Acta 60, 143.

308. Spector, W.G. and Willougby, D.A. (1963) Empire Rheumatism Council Symposium on Salicylates, edit. by A.St.J. Dixon, B.K. Martin et al, p.141, Churchill, London.

309. Suelter, C.H., De Luca, M., Peter, J.B. and Boyer, P.D. (1961) Nature 192, 43.

310. Steel, R.G.D. and Torrie, J.H. (1960) Principles and Procedures of Statistics. McGraw-Hill, N.York.

311. Swaffield, M.N. and Foley, G.E. (1960) Arch. Biochem. Biophys. 86, 219.

312. Swann, M.M. (1955) Proc. Roy. Phys. Soc. Edin. 24, 5.

313. Tager, J.M. and Slater, E.C. (1963) Biochim. Biophys. Acta 77, 227, 246.

314. Ter Well, H.F. and Slater, E.C. (1964) in press.

315. Teply, L.J. (1949) Arch. Biochem. Biophys. 24, 383.

316. Tiedmann, H. and Born, J. (1963) Biochim. Biophys. Acta 78, 217.

317. Trevelyan, W.E. and Harrison, J.S. (1952) Biochem. J. 50, 298.

318. Tyler, D.B. (1950) J. Biol. Chem. 187, 711.

319. Utter, M.F. (1958) Ann. Rev. Biochem. 27, 245.

320. Van Niel, C.B. (1943) Ann. Rev. Biochem. 12, 551.

321. Van Rossum, G.D.V. (1962) Biochem. J. 84, 35P.

322. Wadkins, C.L. (1961) Proc. V. Int. Cong. Biochem. Moscow. p.471. Pergamon Press 1963.

323. Wadkins, C.L. and Lehninger, A.L. (1958) J. Biol. Chem. 233, 1589.

324. Wadkins, C.L. and Lehninger, A.L. (1959) J. Biol. Chem. 234, 681.

325. Wadkins, C.L. and Lehninger, A.L. (1960) Proc. Natl. Acad. Sci. U.S. 46, 1576, 1582.

326. Wadkins, C.L. and Lehninger, A.L. (1963) J. Biol. Chem. 238, 2555.

327. Wallace, W.M. & Lowry, O.H. (1942) J.B.C. 144, 651.

328. Waltner, K., Csernovszky, M. and Kelemen, E. (1959) Biochem.Pharmacol, 2, 50.

329. Warburg, O. and Christian, W. (1935) Biochem. Z. 275, 464.

330. Warburg, O. and Christian, W. (1936) Biochem. Z. 287, 291.

331. Waymouth, C. (1956) J. Nat. Cancer Inst. 17, 315.

332. Waymouth, C. (1959) J. Nat. Canc. Inst. 22, 1003.

333. Webb, J.L., Saunders, P.R. and Thienes, C.H. (1949) Arch. Biochem, 22, 451.

334. Webster, G. (1962) Biochem. Biophys. Res. Comm. 7, 245.

335. Webster, G., Smith, A.L. and Hansen, M. (1963) Proc. Natl. Acad. Sc.U.S. 49, 259.

336. Westfall, B.B., Evans, V.J., Peppers, E.V., Hawkins, N.M. and Earle, W.R. (1956) J. Nat. Cancer Inst. 17, 131.

337. Whitfield, J.G. and Rixon, R.H. (1960) Exp. Cell. Res. 19, 531; 20, 242.

338. Whittam, R. and Bland, D.M. (1964) Biochem. J. 92, 147.

339. Whittam, R., Ager, M.E. and Wiley, J.S. (1964) Nature 202, 1111.

340. Winzler, R.J. and Baumberger, J.P. (1938) J. Cell. Comp. Physiol. 12, 183.

341.

342. Woods, H.G. (1955) Ann. Rev. Physiol. 35, 841.

343. Wu, R. and Racker, E. (1957) Fed. Proc. 16, 274.

344. Ziegler, D.M., Green, D.E. and Doeg, K.A. (1959) J. Biol. Chem. 234, 1916.

345. Ziegler, D.M. Linnane, A.W. and Green, D.E. (1958) Biochim. Biophys. Acta 28, 524.

346. Zwartouw, H.T. and Westwood, J.C.N. (1958) Brit. J. Exp. Pathol, 39, 529.

347. Earle, W.R. (1943) J. Nat. Cancer Inst. 4, 165

348. Tritsch, G.L. and Moore, S.E. (1962) Exp. Cell Res. 28, 360

349. Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1957) Manometric Techniques. Burgess Publishing Co. Minneapolis.